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Characterization of nuclear orphan receptors NR4A in inflammatory signaling in human mast cells and their modulation by adenosine receptors

PhD Thesis submitted by

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Table of Contents

Abbreviations

AC	Adenylyl Cyclase
ADA	Adenosine Deaminase
ADP	Adenosine Diphosphate
AML	Acute Myeloid Leukemia
AMP	Adenosine Monohosphate
ATP	Adenosine Triphosphate
AR	Adenosine Receptor
BAL	Bronchoalveolar Lavage
bFGF	basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CGS-21680	9-Chloro-2-(2-Furanyl)-[1,2,4]Triazolo[1,5-C]Quinazolin-5-Amine
CHOP	C/EBP Homologous Protein
COPD	Chronic Obstructive Pulmonary Disease
CoREST	Co-Repressor for Element-1-Silencing Transcription Factor
CREB	cAMP Response Element Binding Protein
CREM	cAMP Response Element Modulator
Ct	Threshold Cycle
CNS	Central Nervous System
DAPI	4’,6-Diamidino-2-Phenylindole
DBD	DNA Binding Domain
DNA	Desoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ERK1/2	Extrancellular Signal-Regulated Kinase 1/2
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GPCR	G Protein-Coupled Receptors
HMC-1	Human Mast Cell Line-1 (Human Mast Cell Line)
IB-MECA	1-Deoxy-1-[6-[[(3-Iodophenyl)Methyl]Amino]-9h-Purin-9-Y L]-N Methyl-B-D-Ribofuranuronamide
IL	Interleukin
IP3	Inositol 1,4,5-triphosphate
LAD-2	Laboratory Of Allergic Diseases-2 (Human Mast Cell Line)
LAMP1	Lysosomal-Associated Membrane Protein 1
LBD	Ligand Binding Domain
JNK	C-Jun N-Terminal Kinase
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
LUC	Luciferase
MAPK	Mitogen-Activated Protein Kinase
MIP-1α	Macrophage Inflammatory Protein-1 Alpha
NBRE	Nerve-Growth Factor Responsive Element
NECA	5’-N-Ethylcarboxamido-Adenosine

NR4A	Nuclear Orphan Receptor 4 A
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-Buffered Saline
PERK	Protein Kinase-Like Endoplasmic Reticulum Kinase
PI3K	Phosphoinositide-3-Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
POMC	Pro-opiomelanocortin
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SAPK	Stress-activated Protein Kinase
SCH-58261	7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine)
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
SMRT	Silencing Mediator of Retinoid and Thyroid Hormone Receptor
TBS	Tris-buffered Saline
TBST	Tris-buffered Saline Tween 20
UT	Untreated
VEGF	Vascular Endothelial Growth Factor

1 - Summary

Adenosine is a paradoxical inflammatory modulator, which can contribute both to inflammatory shutdown and to the persistence of inflammation. In one hand, adenosine acts as an anti-inflammatory molecular by inhibiting several immune cell functions. On the other, the concentration of adenosine is potent increased in the bronchoalveolar lavage (BAL) of asthmatics patients [1]. Adenosine mediates its biological effects through activation four G-protein coupled adenosine receptors (ARs), namely the A_1, A_{2A}, A_{2B} and A_3 adenosine receptors. These four types of AR differ in their affinity to adenosine, in the type of G protein they recruit and their differentially expressed in different cells and organs. Although some of the functions of the ARs have been characterized in animal models, cellular effectors of adenosine have remained largely unknown. The aim of this work was, first, to identify AR-subtype specific downstream cellular factors that mediate adenosine's pro- or anti-inflammatory effects and, second, to characterize the interplay between the anti-inflammatory A_{2A}AR and other inflammatory stimuli.

In the first part of this thesis, we employed a transcriptomics approach for the identification downstream effector mechanisms of ARs. By combining selective AR activation with genome wide transcriptional screening, we were able to establish a novel link between adenosine inflammatory signaling and the nuclear orphan receptor 4A family of transcription factors. The central finding of this study was that the concomitant treatment with the A_{2A}AR antagonist SCH-58261 significantly enhanced the effect of the adenosine analogue NECA on NR4A2 and NR4A3 induction. Further, we characterized the intracellular signaling pathways associated with the upregulation of these factors, and established that activation of ERK1/2 kinases downstream of PKC mediated NR4As induction by AR, at least partially. Given the multiple roles of these transcription factors, adenosine's effect on NR4A2 and NR4A3 could have broad pathophysiological implications.

The second part of this thesis was aimed at analysing the effect of A_{2A}AR activation on other inflammatory stimuli, namely combination of phorbol 12-myristate 13-acetate, PMA (P) and the calcium ionofore Ionomycin (I), in LAD-2 human mast cells. This study allowed us to characterize several aspects of NR4A2 signaling in activated mast cells. First, we observed a strong upregulation of NR4A2 coupled to phosphorylation-dependent ubiquitination, a previously unknown post-translational modification of NR4A2. Second, cell fractionation and immunofluorescence experiments showed that P/I-induced phosphorylation-dependent ubiquitination led to an accumulation of NR4A2 in the endoplasmic reticulum and in the endolysosomal compartment 6 h after treatment, and that inhibition of the proteasome with MG132 increased the accumulation of NR4A2 in these organelles. Third, we established IL-4 as a downstream effector of NR4A2, which was largely dependent on the PKC and ERK kinases and that could be reverted NR4A2 silencing. Fourth, we observed that proteasome inhibition through MG132 pretreatment enhanced P/I-induced IL-4

release 6 h after treatment (in correlation with the accumulation of NR4A2 in cytoplasmic organelles), but that strongly reduced IL-4 release, despite NR4A2 stabilization. These observations suggested the late release of IL-4 could depend on another factor, and since proteasome degradation of IκB is required for the activation of NF-κB, we reasoned that this signaling pathway could be involved in late phase IL-4 release. In fact, pretreatment with the NF-κB inhibitor BAY 11-7082 significantly inhibited the release of this cytokine at a late time point and MG132 also inhibited NF-κB activation, strongly suggesting that the late inhibitory effect of MG132 on IL-4 release could be mediated by inhibition of NF-κB signaling. Furthermore, NR4A2 silencing partially reduced NF-κB-LUC reporter activity, suggesting that NR4A2 is required for the activation NF-κB at a later time-point. These results indicate that while NR4A2 is the central transcriptional factor driving IL-4 release in the early phase (6 h), NF-κB becomes determinant factor in IL-4 synthesis later time point, explaining the biphasic effect of proteasome inhibition. Finally, we showed an inhibitory effect of the adenosine receptor A_{2A} on NR4A2 and NF-κB dependent IL-4 induction, suggesting that through the modulation of IL-4 release, A_{2A}AR activation in mast cells can negatively influence the development of allergic Th2 responses.

2- Introduction

2.1 Orphan nuclear receptor 4A family: NR4A

The orphan nuclear receptor 4A family (NR4A) belongs of nuclear hormone receptor superfamily. These receptors were first recognized based on their resemblance to classical nuclear receptors and their ligands were unknown [2]. It consists of three members, NR4A1 (also known as the nerve growth factor inducible B, NGFI-B, Nur77, TR3, N10, DHR38, NAK-1, TIS1), NR4A2 (nur-related factor 1, Nurr1, TINUR, NOT, RNR-1) and NR4A3 (neuron derived orphan receptor 1, Nor1, MINOR, TEC, CHN) [3-5]. NR4A1 was found to be induced when cells were treated with the nerve growth factor, and NR4A2 and NR4A3 were cloned due to their resemblance to other nuclear receptors [4, 5]. Based on their sequence homology, NR4A1, NR4A2 and NR4A3 were suggested to form a subfamily [2, 4, 5].

2.1.1 Structure of NR4As

The members of NR4A group are high homology in the DNA binding domain (DBD) and C-terminal ligand binding domain exhibit ~91-95% and ~60% homology, and moderate in the ligand binding domain (LBD), but the N-terminus has more differences [6] (Figure 1).

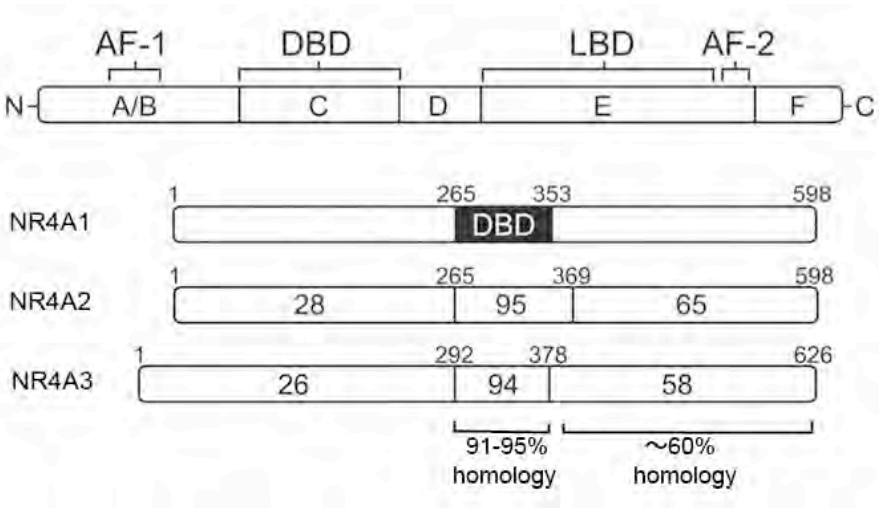

Fig. 1 Domain structure of NR4A orphan receptors (Adapted from [7])

The crystal structures of NR4A2 LBDs have been resolved and reveal that both of them consist of 12 helices and that the ligand-binding pocket is loaded with bulky side chains from residues which are conserved in the family (Figure 2). This implies

that these receptors do not exclude the existence of endogenous or synthetic ligands, but also advises that activation of NR4As may be ligand-independent [8, 9].

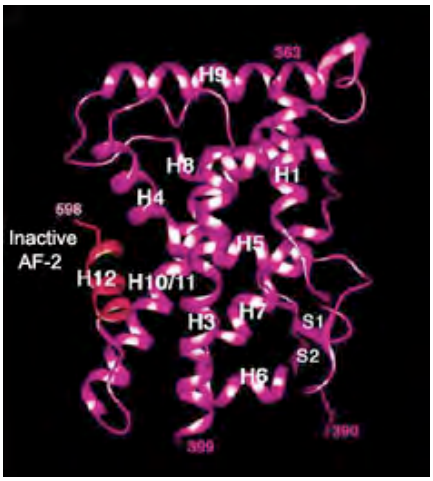

Fig. 2 Crystal structure of NR4A2 LBD. It contains a three-layered antiparallel α-helical sandwich formed by 12 α-helices (H1–H12) and one β-sheet of two strands (S1, S2). The AF2 helix (H12) is shown in red. (from Protein Data Bank：
http://www.rcsb.org/pdb/explore/jmol.do?structureId=1OVL&bionumber=1 [8])

2.1.2 NR4As expression and localization

NR4As are widely expressed in various tissues, such as lung, brain, liver, skeletal muscle, blood vessels, adipose tissues and immune cells [10]. The expression of the receptors of the NR4A subfamily is strongly induced by external stimuli. NR4As are classified as early response genes (stress response genes) with maximum induction within 2 h after stimulation in immune cells such as mast cells [11]. The range of physiological signals that induce the expression of NR4A receptors is wide, from fatty acids and peptide hormones to stress and magnetic field [10]. The activation of NR4A receptor is dependent on several kinase pathways, including PI3K/Akt, PKA and MAPK pathways [12-14]. NR4A2 promoter also contains the binding sites for these transcription factors [15].

NR4A receptors are usually located in the nucleus [29]. Katagiri et al. have studied the pathways mediating the localization of NR4A1. They have demonstrated that in NR4A1, there are two nuclear localization signal sequences in the DBD and three nuclear export signal sequences in the LBD. They have shown that the nerve growth factor can induce NR4A1 relocalization to the cytoplasm by phosphorylating serine 105. This phosphorylation was shown to be regulated by the MAPK pathway. In their studies, the nerve growth factor also induced relocalization of RXR as a heterodimer with NR4A1 [30]. Relocalization of NR4A1 to cytoplasm has also been observed in response to apoptotic factors [31]. Cytoplasmic NR4A1 was found to associate with mitochondria which led to depolarization of the mitochondrial membrane and release of cytochrome c [31]. It was further demonstrated that these events were the result of interaction with an anti-apoptotic protein Bcl-2 and that the interaction changed the

nature of Bcl-2 from anti- to pro-apoptotic [32]. It seems that the cytoplasmic NR4A1 has functions that are distinct from the nuclear NR4A1.

2.1.3 NR4As transcriptional activity

NR4A family members bind as monomers to the consensus DNA sequence AAAGGTCA. This element is termed NBRE (NGFI-B response element) (Figure 3). The A-box of the DBD is responsible for recognizing the AA sequence preceding the classical response element of non-steroidal nuclear receptors (AGGTCA) [16, 17]. NR4A1, NR4A2 and NR4A3 have been shown to bind DNA as homodimers and as heterodimers with each other on the NurRE (nur-response element, AAAT(G/A)(C/T)CA) (Figure 3), which is an inverted repeat of two slightly converted NBRE half-sites [18]. Heterodimers formed within the NR4A family also bind NurRE much more efficiently than monomeric or homodimeric NR4As [18] (Figure 3). Maybe differential expression of the NR4A receptors can influence the abundance in which the highly active and less active NR4A heterodimers are present in the cell. In addition, the amounts of homodimers and monomers may be affected. The promoters of different genes can be activated depending on the expression levels of NR4As. Gene activation is also determined by the differential activity of NurRE and NBRE on target genes. Moreover, NR4A1 and NR4A2 (not NR4A3) bind DNA as heterodimers while using RXR on DR5 elements. NR4A2/RXR heterodimers can also be formed on NBREs without RXR binding to DNA [19-21]. Activation or suppression genes by NR4As have been investigated and the list of genes is extensive [22]

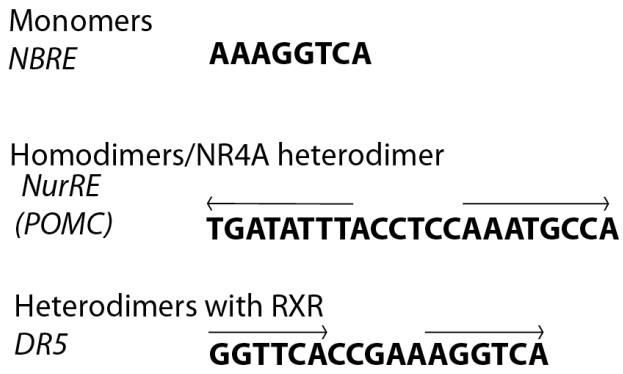

Fig. 3 NR4A response elements (Adapted from [10])

The transcriptional activity of NR4As can also be regulated by co-repressors and co-activators. NR4A-mediated trans-activation (LBD) involves unusually active N-terminal AF-1 domains that mediate coactivator recruitment [23]. Silencing mediator of retinoid and thyroid hormone receptor (SMRT) binds to NR4A2 LBD, and has been suggested to function as a coregulator surface in NR4A1 [9]. This binding site has been shown to bind both corepressors and coactivators [24, 25]. However, the differences in coactivator and corepressor binding compared to other nuclear

receptors are likely to explain partially the constitutive activity of the NR4A receptors and the permissive nature of NR4A1/RXR and NR4A2/RXR heterodimers.
In addition to dimerization and cofactors, several mechanisms have been described to control the transcriptional activity of the NR4A receptors. NR4As are regulated by phosphorylation. Phosphorylation can either enhance or repress the transcriptional activities of the NR4A receptors. For example, the MAPK pathway phosphorylates NR4A1 and enhances its transactivation on POMC (pro-opiomelanocortin) promoter [12]. Phosphorylation of serine 350 in the NR4A1 DBD has been shown to decrease its binding to NBRE [26], thus offering another possible target for regulation by phosphorylation. NR4A2 has also been shown to possess two putative sumoylation sites at lysines 91 and 577. Mutational analyses have shown that sumoylation at position 91 can repress the transcriptional activity of NR4A2, and sumoylation at position 577 can enhance it [27]. Post-translational modifications could thus provide an important ways of controlling the activity of orphan nuclear receptors. It demonstrated ubiquitination and degradation of NR4A1 were triggered by JNK activation [28]. Methylation of the receptors of the NR4A family has not been reported.

2.1.4 Biological functions of NR4As

NR4A receptors therefore have varied functions in controlling multiple physiological events. Mice with targeted deletion of the NR4A2 gene die after birth, probably due to problems with the control of respiratory functions [33]. NR4A1, NR4A2 and NR4A3 could be induced by physiological and physical stimuli, including inflammatory cytokines, growth factors, calcium phorbol esters, prostaglandins, fatty acids, growth factors, neurotransmitters, hormonally-active peptides, and other cellular stressors including fluid sheer stress, magnetic fields, and membrane depolarization [10]. Activation of NR4A receptors by multiple stimuli is consistent with the increasing number of tissue-specific receptor-mediated effects that are important for their function [10].

NR4As and inflammation

The extensive roles of NR4A1, NR4A2 and NR4A3 in inflammation, vascular function, metabolic processes and central nervous system (CNS) have been reviewed. NR4A knockout animal models have provided insights into a few of the endogenous functions of NR4A receptors. However, some redundancies among these receptors (particularly NR4A1 and NR4A3) may mask specific functions in mouse models [34, 35]. The first evidence linking NR4A expression with inflammatory signaling was reported by Woronicz et al., who noted that NR4A1 is induced in apoptotic T-cells and that inhibition of NR4A1 function prevented apoptosis [36, 37]. However, knockdown of NR4A1 in mice exhibits unimpaired T-cell apoptosis, and functional redundancy of NR4A1 and NR4A3 in T-cell apoptosis has been reported [38, 39]. NR4A2 expression has elevated levels of inflammatory arthritis and can be

downregulated with glucocorticoids [35]. Recently study has shown that NR4A2 induces Foxp3 and regulates differentiation of $CD4^+$ T cells, indicating that NR4A2 is able to maintain T-cell homoeostasis [40]. Much like T-cells, NR4A1 expression is increased in apoptotic macrophages and, contrary to the experiments performed in T-cells, peritoneal macrophages isolated from NR4A1 deficient mice reveal a phenotype of reduced cell death [41]. Responding to inflammatory activation, all NR4A receptors are potently induced in macrophages [42, 43]. This inducible expression of NR4A receptors in macrophages depends on the activation of NF-κB signaling, as exemplified by the recruitment of NF-κB to response elements in the NR4A1 promoter [42]. Functional studies have established that NR4A receptors both activate and repress inflammatory genes in macrophages [43-45]. Microarray analysis of macrophages overexpressing NR4A1 showed increased pro-inflammatory gene expression [44]. Interestingly, among the identified direct NR4A1 target genes was the inducible kinase IKKi, which functions as a NF-κB activating kinase, providing a potential mechanism for the activation of inflammatory gene expression by NR4A1 in macrophages [45]. In contrast, another study showed over-expression of each NR4A member reduces certain inflammatory genes (i.e. IL-1α, IL-6, IL-8, MIP-1α and MCP-1) and the uptake of oxidized low density lipoprotein (LDL) [43]. Finally, it has recently been shown that NR4A2 transcriptionally represses the inflammatory genes TNF-α, iNOS, and IL-1α in a mouse macrophage cell line [45]. This repression was mediated through NR4A2-dependent recruitment of the co-repressor for element-1-silencing transcription factor (CoREST) complex to the target promoter and the subsequent clearance of NF-κB [45].

NR4As and cancer

NR4As expression has been reported in multiple cancer cell lines [46, 47]. Higher level of NR4A1 was found in colon and pancreatic tumors in patients [48, 49] Cytoplasmic and nuclear NR4A2 was found in cohort of bladder cancer patients, and it was shown that cytosolic and nuclear NR4A2 staining was higher in patients compared with healthy donors, moreover, NR4A2 levels were associated with increasing histopathological grade of the tumor [50]. NR4A1, NR4A2 and NR4A3 protein expression was variable with different types of cancers, and they could have prognostic significance for patients with tumor. Studies with overexpression of NR4A or NR4A knockout animal models provided valuable insights some insights into the function of NR4As in cancer cells. Knockdown NR4A1 resulted in induction of apoptosis, inhibition of cell proliferation and decreased angiogenesis, indicating NR4A1 is a pro-oncogenic factor in cancer [48, 49]. Overexpression of NR4A1 in lung cancer cells enhances proliferation and cell cycle progression [51]. The knockdown effect of NR4A2 in bladder cancer cells did not affect cell proliferation, but decrease cell migration, suggesting NR4A2 was anti-apoptotic factor [50]. These results reveal some differences between breast cancer cells and other cancer cell lines. It was also shown that NR4A1/NR4A3 double knockout mice rapidly developed lethal acute myeloid leukemia (AML) and died 2 to 4 weeks after birth [52]. Loss of

both orphan nuclear receptors was accompanied by dysregulation of multiple pathways and this included enhanced production of hemapoietic stem cells. These results suggested a potential role for these receptors in leukemia. In agreement with this hypothesis, it was reported that both NR4A receptors were also decreased in leukemia patients [52]. In conclusion, NR4A1, NR4A2 and NR4A3 play different roles in cancer. These properties can potentially be utilized as targets for cancer therapy.

2.2 Adenosine and adenosine receptors

2.2.1 Adenosine metabolism

The endogenous nucleoside adenosine plays a central role as a structural element of nucleic acid and in the energy metabolism of all living organisms (Figure 4). Adenosine is a byproduct of ATP degradation, acting as a sensor of the metabolic state, in turn modulating biological functions in a variety of cell types, tissue and organs. The physiological effects of adenosine were first described in the cardiovascular system and gastrointestinal tract [53], although modulatory functions in a much broader spectrum of organ systems has been described.

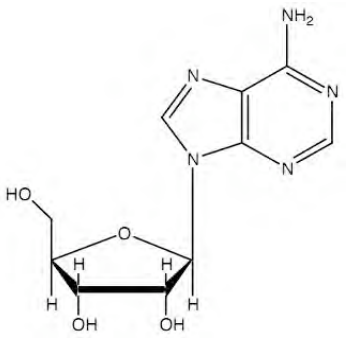

Fig. 4 Adenosine structure

Adenosine concentration is related to energy consumption and under basal conditions is kept within a narrow range (30-300 nM) [54]. During metabolic stress conditions, such as hypoxia or ischemia, extracellular levels of adenosine can reach a concentration of 30 μM or higher [55, 56].

Adenosine arises mainly from the cleavage of adenosine monophosphate (AMP) by either intracellular or extracellular 5’ nucleotidase or efficient equilibrative

transporters balance intracellular and extracellular adenosine level. S-adenosyl homocysteine has also been reported to be a minor pathway of adenosine formation [57] (Figure 5) Adenosine degradation involves either phosphorylation by adenosine kinase or deamination to inosine by adenosine deaminase, respectively [58, 59]. Adenosine deaminase also degrades 2'-deoxy adenosine, which is a lymphotoxic product of the purine metabolism [60].

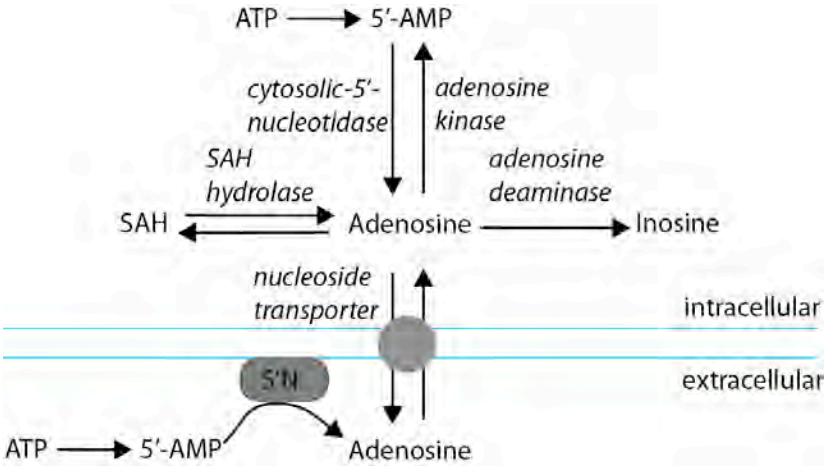

Fig. 5 Metabolic pathway of adenosine (Adapted from Adair, 2005) [61]

2.2.2 Adenosine receptors

Adenosine binds four different ARs: A_1, A_{2A}, A_{2B} and A_3, which belong to G protein-coupled membrane receptors (GPCRs) (Figure 6). Adenosine receptors have a variable distribution in different tissues (central nervous system, cardiovascular, renal, respiratory, immune and gastrointestinal), where they modulate biological functions [62].

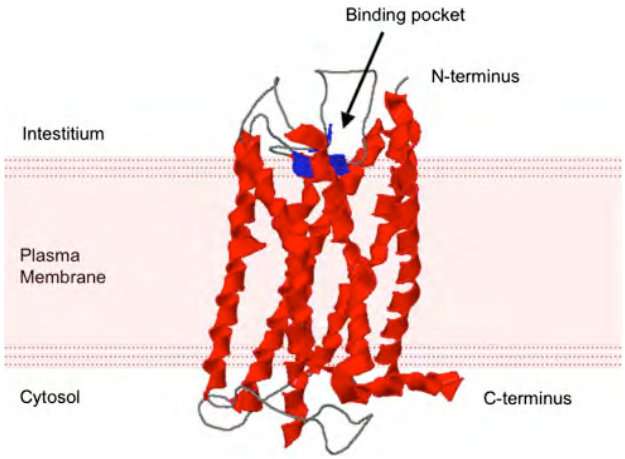

Fig. 6 Adenosine receptor [63]

The four AR subtypes are encoded by distinct genes and have different

pharmacological profiles in regard to agonist and antagonist binding properties. A_1AR and A_{2A}AR are activated by low levels of adenosine (0.01 to 1 μM), whereas A_{2B}AR and A_3AR require higher amounts (>10 μM). All of the AR subtypes can be characterized according to the affinity to the natural agonist adenosine. In physiologic conditions the rank order of potency for adenosine is as follows: $A_1 \geq A_{2A} >> A_3 \approx A_{2B}$ [64]. However, in artificial systems overexpressing ARs, A_3AR can also be activated by low adenosine concentrations [64].

The affinity of various AR agonists and antagonists at the AR subtypes are summarized in Table 1.

Table 1. Binding affinity of agonists and antagonists at human adenosine receptor subtypes (adapted from [65, 66])

	Compound	*K*i value for AR (nM)			
		A_1AR	A_{2A}AR	A_{2B}AR	A_3AR
Agonist	CPA	**2.3**	794	18,600	72
	Selodenoson	**1.1**	306	N.D*	N.D
	CGS21680	289	**27**	>10,000	67
	CV-3146	>10,000	**290**	>10,000	>10,000
	BAY-60–6583	>10,000	>10,000	**4**	>10,000
	IB-MECA	51	2,900	11,000	**1.8**
	CP-608039	7,200	N.D	N.D	**5.8**
Antagonist	DPCPX	**3.9**	129	56	3,980
	FR194921	**2.9**	>10,000	N.D	>10,000
	SCH 58261	725	**5.0**	1,100	1,200
	SCH 442416	1,100	**0.048**	>10,000	>10,000
	MRS1754	403	503	**2.0**	570
	MRE 2029-F20	245	>1,000	**3.0**	>1,000
	OT-7999	>10,000	>10,000	>10,000	**0.61**
	MRS3777	>10,000	>10,000	>10,000	**47**

*: Not determined
Figures in bold indicate the receptor with the highest affinity

2.2.3 Adenosine receptor signaling and regulation

The four subtypes AR couple to an intricate network of G proteins (Table 2), which enable the endogenous modulator adenosine to communicate with cells in complex manner. Table 2 summarizes the chromosomal location, length G-protein partners and downstream signaling by each receptor subtype.

Table 2. Properties of human adenosine receptor subtype

AR Subtype	Genebank Number	Chromosomal Location	Length (Amino Acids)	G-protein Coupling	Downstream Signaling [67]
A_1AR	S45235	1q32.1	326	i, o, q	↓ cAMP [67] ↑ PLC [68] ↓ Ca^{2+} channels [69] ↑ K^+ channels [70]
$A_{2A}AR$	S46950	22q11.2	412	s, olf	↑ cAMP [71] ↑ PLC [71] ↑ IP_3 [72]
$A_{2B}AR$	X68487	17p11.2-12	332	s, q	↑ cAMP [73] ↑ IP_3 [73] ↑ PLC [74]
A_3AR	L22607	1p13.3	318	i, o	↓ cAMP [75] ↑ Ca^{2+} channels [76] ↑ PLD [77] ↑ PLC [78]

A_1AR

A_1ARs were found in several species including human, dog, rat, cow and rabbit A_1AR [79]. It has been demonstrated that the A_1AR can not only inhibit adenylate cyclase (AC) by interacting with Gi or Go proteins (Table 2), but can also inhibit G protein-coupled activation of voltage dependent Ca^{2+}-channels and induced phospholipase C (PLC) activation via Gq protein [68]. It determines the activation of the family of extracellular signal-regulated kinase 1/2 (ERK 1/2), thus leading to pro-inflammatory response [69]. Meanwhile, A_1ARs are involved in responses that reduce oxygen demand by directly decreasing heart rate, force of contraction and conduction of action potential (through opening of K^+-channels) as well as by indirect antiadrenergic effects (through inhibition of AC) [80] (Figure 7).

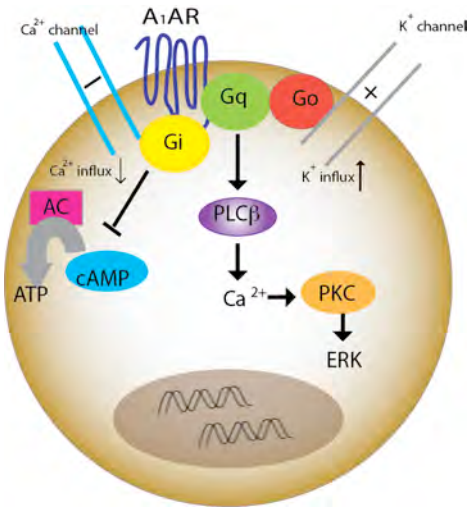

Fig. 7 Adenosine A_1 receptor signal transduction pathway [81, 82]

In heart, selective A_1AR agonist has a potential to control arrhythmia, such as Selodenoson, which influences on heart rate without lowering blood pressure [83]. It has been tested in Phase II clinical trials for its capacity to slow heart rate in atrial fibrillation. In CNS, A_1AR agonist, GW-493838, was found to inhibit electrically induced nociception-specific blink reflex responses [84]. In endocrine system, A_1AR agonist GR79236 has been tested in humans for adjuvant therapy in insulin resistance (type 2 diabetes) [85, 86]. A_1AR-selective antagonist FR194921 had been found to have ability in the treatment of dementia and anxiety disorders [85, 86]. A_1AR antagonists are effective diuretic agents that are useful in treating fluid-retention disorders, in renal system, A_1AR antagonist, BG9719 was for the treatment of acute renal disorders in patients with congestive heart failure [87].

A_{2A}AR

The A_{2A}ARs are expressed in mast cells, T cells, neutrophils, CNS, and vascular smooth muscle cells [88]. It has been shown that adenosine exerts predominantly effects on the immune system, also A_{2A}AR molecule has been identified as an important endogenous immunosuppressing regulator as it has been observed that lung A_{2A}AR activate a protective mechanism in the downregulation of inflammation and tissue damage in different models [89].

A number of effects described activation of A_{2A}AR seems to affect multiple aspects of the inflammatory process, modulating neutrophil activation and degranulation, oxidative species production, adhesion molecules expression, cytokine release and mast cell degranulation [90]. Moreover, A_{2A}AR agonist have been proposed to control inflammation and inflammation-related tissue injury [91, 92]. Despite the beneficial aspects of quenching inflammation the described A_{2A}AR-mediated down-regulation of the immune response is potentially dangerous on its own. Indeed, the premature inhibition of immune cell function may allow pathogens to survive and as a result the overall damage to the organism. On the other hand, the lack of A_{2A}ARs signaling may result in excessive damage with important biological consequences. Therefore, the level of the activation of A_{2A}ARs is the key issue for the balance between the requirement to destroy invading pathogens and the desire to protect host tissue from excessive damage. Manipulation of inflammatory processes may include not only efforts to inhibit inflammation, but also the development of approaches to enhance local inflammatory processes [93].

A_2ARs are coupled to the intracellular transduction pathway through Gs proteins and stimulate the activation of AC resulting in the elevation of intracellular cAMP [88, 94]. A_{2A}AR also activates mitogen-activated protein kinase (MAPK) activity. Adenosine agonists through activation of ERK1/2 using the cAMP-ras-MEK1 pathway, exert mitogenic effects on human endothelial cells via the A_{2A}AR subtype [69]. A_{2A}AR triggers the activation of PKA and subsequently activation of cAMP responsive element-binding protein (CREB), which in turn inhibits NF-κB [95]. Moreover, A_{2A}AR can also induce SOCS-3 expression through EPAC-1 in a cAMP dependent manner [95]. However, the signaling pathways used by A_{2A}AR seem to

vary with the cellular background and the signaling machinery of each cell type (Figure 8).

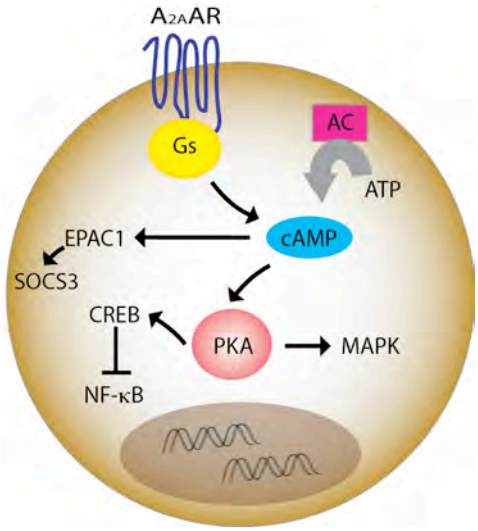

Fig. 8. Adenosine A_{2A} receptor signal transduction pathway (Adapted form Fredholm et.al., 2007) [96]

As a potent anti-inflammatory player, A_{2A}AR agonist can be used in pulmonary disorders and inflammation. Activation of the A_{2A}AR by CGS21680 produces potent anti-inflammatory activity in the allergic asthma rat model [97]. ATL-146e, another selective agonist of the A_{2A}AR can protect mouse liver from reperfusion injury, and the protection effect is reversed by the A_{2A}AR antagonist ZM241385 [98]. Furthermore, the A_{2A}AR agonist ATL-146e has also shown potential for the treatment of sepsis [99] and inflammatory bowel disease [100]. Selective activation of the A_{2A}AR is able to control ulcer formation and inflammation [101]. In heart, selective A_{2A}AR agonist binodenoson (WRC-0470) is tested in Phase III clinical trials and seems to be well tolerated as a coronary vasodilator [102]. In addition, an A_{2A}AR antagonist, KW-6002 (istradefylline), has shown potential in a recently completed Phase II clinical trial (now in Phase III trials) as a novel treatment for Parkinson's disease [103].

A_{2B}AR

The A_{2B}AR, although structurally closely related to the A_{2A}AR and able to activate AC, is functionally very different. It has been postulated that this subtype may utilize signal transduction systems other than AC because of these functional differences [104]. A_{2B}AR has been enigmatic due to the lack of good selective high affinity agonists and antagonists. The A_{2B}ARs, on the contrary with the other adenosine receptor subtypes, are characterized by a low density and it needs high adenosine levels to their activation.

A_{2B}AR has been identified in different tissues and to determine their function, including in the brain, human bronchial epithelium, endothelial cells, muscle cells,

neurons, glial cells, fibroblasts and mast cells [105]. A_{2B}ARs predominantly expressed in human microvascular cells, modulate expression of angiogenic factors [106]. A_{2B}AR subtype appears to mediate the actions of adenosine to increase growth factor production (i.e. VEGF) and cell proliferation of human retinal endothelial cells [107]. In the immune system, A_{2B}AR activation has been documented to be involved in mast cell degranulation and cytokine release, which are the major triggers of asthma [108]. In heart, adenosine causes inhibition of cardiac fibroblasts growth and of vascular smooth muscle cells by activating A_{2B}AR [109, 110]. It is interesting to note that, in contrast to smooth muscle cells, A_{2B}AR induces growth of endothelial cells [111, 112]. Moreover, A_{2B}ARs have been implicated in the regulation of gene expression, intestinal function, neurosecretion and vascular tone [113].

Gs-mediated A_{2B}AR signaling can increase cAMP level leads to activate not only MAPK but also p38 and stress-activated protein/Jun kinase (SAPK/JNK) signaling pathway [69]. In most cell types A_{2A}AR subtype inhibits intracellular Ca^{2+} levels whereas A_{2B}AR, via Gq proteins are linked with the stimulation of PLC and induces Ca^{2+} increase [108]. These two pathways can be activated simultaneously, which enables additional levels of fine-tuning of cellular process (Figure 9).

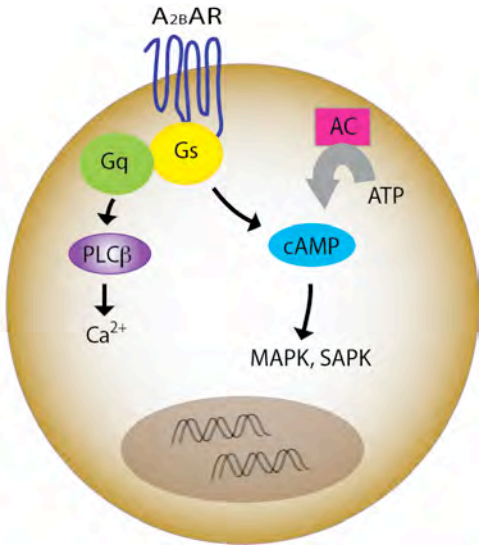

Fig. 9 Adenosine A_{2B} receptor signal transduction pathway (Adapted form Hasko et. al., 2009)[114]

In asthmatic lung, selective antagonist for the A_{2B}AR, could have therapeutic potential in asthma treatment [115] In addition, theophylline and other xanthines might have anti-asthmatic effects by blocking A_{2B}AR, although the evidence for this is still controversial due to the lack of a property animal model. A_{2B}AR antagonists are also under consideration for diabetes treatment [116]. So far there is no commercial A_{2B}AR-selective agonists available, however, recently, A_{2B}AR-knockout mice have hampered further clarification of the functional significance of this receptor.

A_3AR

In comparison with the other adenosine receptors, A_3AR exhibits large differences in structure, tissue distribution and its functional and pharmacological properties among species [117]. However, marked differences exist in expression levels within and among species [118]. The classical pathways associated with A_3AR activation are the inhibition of AC activity, through the coupling with Gi proteins, and the stimulation of PLC, inositol triphosphate (IP3) and intracellular Ca^{2+}, via Gq proteins [69, 119]. In addition, in different recombinant and native cell lines, A_3AR is involved, like the other adenosine subtypes, in the modulation of MAPK activity [120] (Figure 10). The functional role of this AR subtype remains controversial, mainly due to interspecies difference. In rat, mast cell degranulation seems to be dependent on A_3AR activation and A_3AR antagonism attenuates pulmonary inflammation, reduces eosinophil infiltration and decreases airway mucus production. In contrast, A_3AR agonists IB-MECA may be a new family of orally bioavailable drugs to be developed as potent inhibitors of autoimmune-inflammatory diseases [121].

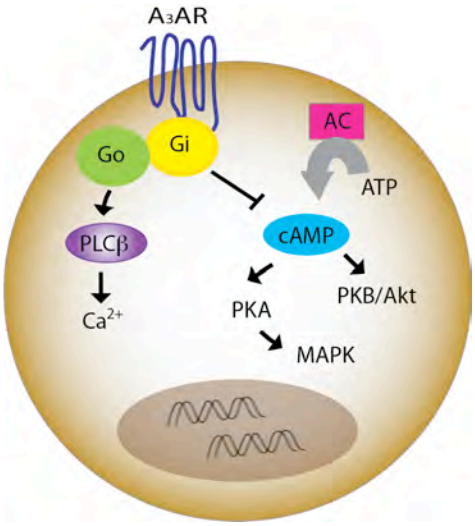

Fig. 10 Adenosine A_3 receptor signal transduction pathway (Adapted from Fishman et.al.,2012) [122]

It was shown that the A_3AR is more highly expressed in tumor than in normal cells, which may justify A_3AR as a potential target for tumor growth inhibition [123]. A_3AR activation has been implicated in inhibition of tumor growth both in vitro and in vivo [122], and selective A3AR agonist IB-MECA is in clinical trials for colon carcinoma. In heart, The A_3AR agonist CP608039 is in development for use in perioperative cardioprotection [124], and the A_3AR agonist Cl-IB-MECA protects rat cardiac myocytes from the toxicity induced by the cancer chemotherapeutic agent doxorubicin [125].

2.2.4 Biological functions of adenosine

In many tissues and organs, adenosine acts as a chemical messenger with varied functions.

A role for adenosine in pulmonary disease was first described in the late 1970s when it was discovered that adenosine and related synthetic analogues were potent enhancers of IgE-dependent mediator release from isolated rodent mast cells [126]. A couple years later, adenosine administered by inhalation was shown to be a robust bronchoconstrictor of asthma patient but, importantly, not of normal airways [127]. Further work indicated that both allergic and non-allergic asthmatics responded in a similar way and that the effect was also seen with AMP, ADP and ATP [128].

First, Increased formation of adenosine occurs in chronically inflamed airways. Adenosine as well as ARs are intimately involved in defining the pathophysiology of asthma and chronic obstructive pulmonary disorder (COPD). Adenosine receptors on immune cells included in the lung seem to have particularly crucial roles. Most compelling are the observations that inhaled adenosine can induce bronchoconstriction in patients suffering from asthma or COPD, but not in healthy individuals, and AR blockade can prevent this bronchoconstrictive response [129]. Second, expression of the four identified ARs has been shown in a large number of proinflammatory cells as well as in the peripheral lung parenchyma of patients with COPD. This basic concept of AR activation on mast cells has been confirmed by studies using animal models of asthma. These studies have also identified A_{2B}AR [130] and A_3AR [131, 132] as the main contributors that mediate the stimulatory effect of adenosine on mast-cell activation. Third, beside mast cells, pro-inflammatory effects of A_{2B}AR stimulation have also been observed with human bronchial smooth muscle cells [133], human bronchial epithelial cells [134] and human lung fibroblasts [135], which respond to adenosine by increased release IL-19 [134]. A_{2B}AR activation on human lung fibroblasts promotes their differentiation into myofibroblasts that are capable of overproducing extracellular matrix [135], which suggests that adenosine may participate in the fibrosis and remodeling of the lung during asthma and COPD. Furthermore, increased A_{2A}AR agonists have been found to decrease permeability of microvascular and inhibit release of chemotactic cytokines in lung inflammation. Mice lacking A_{2A}AR was shown enhanced lung inflammation [136]. In addition, using A_{2A}AR knockout mice, the A_{2A}AR-cAMP axis was identified as a potent endogenous anti-inflammatory signaling pathway that reduces airway reactivity and inflammatory cell migration [137]. Thus, A_{2A}AR agonists appear to be effective at curbing inflammatory lung tissue damage.

In CNS, adenosine acts as a neuromodulator, integrating different brain functions. It can modulate CNS excitability at different levels and play a role in mechanisms of seizure susceptibility [138], cerebral blood flow [139], pain perception [140], basal ganglia function [141], sleep induction [142] and respiration [143].

Adenosine has a potent function of vasodilation in most vascular beds. This nucleoside is marketed for myocardial perfusion scintigraphy in humans. It has also shown robust angiogenic effects and the main pro-angiogenic actions of adenosine is mediated by the induction of pro-angiogenic factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). Adenosine also protects the myocardium form hypoxia and reperfusion injury through ischemic

preconditioning. Moreover, adenosine shows antiarrhythmic effect [144].
Extracellular adenosine plays a role in kidney by affecting vascular and tubular functions. By activating A_1AR on afferent arterioles, adenosine lowers glomerular filtration rate by constricting afferent arterioles [145]. In contrast, it induces vasodilation in deep cortex and medulla via activation A_{2A}AR [146]. Moreover, adenosine can also inhibit renin release and stimulates NaCl transport [147]. Adenosine has also contributed in glucose metabolism by increasing insulin sensitivity and glucose uptake [85].

Adenosine, whose concentration increases within hypoxic regions of solid tumors, has been recognized able to interfere with the recognition of tumor cells by cytolytic effector cells of immune system [148]. Each of its receptor subtypes has pro- and anticancer effects. It appears that all the ARs are possible targets for the development of novel approaches to the treatment of cancer. The antitumorigenic role of A_1AR in cancer was mainly studied in A_1AR knockout mice, demonstrating that activation of the A_1AR on microglia inhibits the growth of glioblastomas [149]. As for A_{2A}AR, it has been suggested that this subtype blocks antitumor immunity. In the tumor environment of hypoxia and high adenosine levels, activation of A_{2A}ARs leads to immunosuppressive effects [150], which decreases antitumor immunity and thus encourages tumor growth. The role of the A_{2B}AR in cancer is characterized as pro-cancer player. Activation of A_{2B}ARs leads to the release of angiogenic factors that promote tumor growth [151, 152]. Adenosine accumulates at high levels in hypoxic regions of solid tumors, and several lines of evidence indicate that the A_3AR is overexpressed in several solid tumors suggesting that it may be a tumor marker [153]. Furthermore it has been reported that it plays a pivotal role in the adenosine-mediated inhibition of tumor cell proliferation [154]. Therefore clinical trials have demonstrated the possibility of using A_3AR agonists for treatment of cancer [155]. In vivo studies have shown that adenosine exerts a profound inhibitory effect on the induction of mouse cytotoxic T-cells, without substantially affecting T-cell viability [156]. The ability of adenosine to specifically inhibit tumor cell growth in vitro and in vivo suggests that the activation and/or blockade of the pathways downstream of adenosine receptors may contribute to tumor development. Furthermore, the extracellular adenosine concentration may be a crucial factor in determining the cell progression pathway, either in the apoptotic or in the cytostatic state [148].

Alternate roles of adenosine in inflammation

In many tissues and organs, adenosine receptor system has been taken as both a rapid sensor of tissue injury and the major ‘first-aid’ machinery [157]. Adenosine interacts with ARs on immune cells to modulate the inflammatory processes. Animal models of asthma, ischemia, inflammatory bowel disease, sepsis, arthritis and wound healing have shown the regulatory functions of different AR in dictating the development and progression of disease [157]. On one hand, increased concentration of extracellular adenosine represents a potent alarm molecule for reporting tissue. On the other, it

decrease tissue energy demand via a direct inhibitory effect on parenchymal cell function and provides a more favorable cellular environment by increasing nutrient availability via vasodilatation [158]. Adenosine receptors are expressed on different kinds of immune cells, such as mast cells, macrophages, neutrophil and lymphocytes.

A large number of studies show that mast cell mediators, such as cytokines and histamine may play an important role in inflammatory diseases in response to adenosine [157]. Although A_{2A}, A_{2B} and A_3ARs are all expressed on the cell membrane of mast cells [129], there is still not clear which receptors take response for the increased mast cell activation following AR activation. Adenosine can increase the secretion of the major asthma-promoting cytokine IL-13 by wild type mouse mast cells but not A_{2B}AR knockout mouse cells. This result implies that A_{2B}AR is the receptor that involved in pro-inflammatory effects of adenosine, such as cytokine and histamine release [130]. Furthermore, studies using ADA knockout mice suggested that elevated endogenous adenosine induced lung mast cell degranulation by engaging A_3AR [131]. Thus, A_{2B} and A_3AR played different pro-inflammatory roles in primary mouse mast cells. A_{2B}AR stimulation in human HMC-1 cells induces secretion of cytokines IL-4 and IL-13, as well as a number of other pro-inflammatory cytokines such as IL-1β and IL-8. In addition, IL-4 incubation resulted in the increased expression of A_{2B} and reduced expression of A_{2A}AR on human mast cells. These results suggest that Th2 cytokines in the asthmatic lung may alter adenosine receptor expression on airway mast cells to promote increased responsiveness to adenosine [159]. The observation of increased IL-4 and IL-13 production following AR activation strongly implicates human mast cell ARs as important players of human asthma. In chapter 4 of this thesis, new insights about IL-4 release in activated mast cells and the modulatory effect of A_{2A}AR are presented.

In macrophages, the activation of A_{2A}AR can limit tumor necrosis factor α (TNF-α) production following macrophage activation. Several studies agree that the A_{2A}AR is the primary AR subtype that mediates inhibition of TNF-α [160-162]. A key role for A_{2A}AR was discovered when adenosine failed to upregulate *E.coli*-induced IL-10 release by macrophages lacking A_{2A}ARs but not in wild-type macrophages [163]. While A_{2B}AR can be critical to the stimulatory effect of adenosine on IL-6 production, as NECA cannot induce the secretion of IL-6 in A_{2B}AR knockout however, not wild-type macrophages [162].

Adenosine is also a potent modulator of neutrophil functions. Through the activation of ARs in this cell type, this molecule can regulate phagocytosis and the production of reactive oxygen species (ROS) [164, 165]. Also, engagement of A_{2A}AR has been shown to inhibit the production of a range of cytokines including TNF-α, macrophage inflammatory protein 1α (MIP-1α), MIP-1β, MIP-2α and MIP-3α [166]. A_{2A}AR prevents neutrophils from undergoing apoptosis in inflamed site [167-169]. Thus, adenosine can be regarded as a general neutrophil function regulator. Lymphocyte function also appears to be potently regulated by A_{2A}AR. For example, adenosine inhibits IL-2 production in $CD8^+$ T cells, via A_{2A}AR [170]. However, the production of neither IFN-γ nor IL-4 cytokines was influenced by A_{2A}AR activation [78]. In

summary, A_{2A}AR can regulate different inflammatory signaling pathways on different cell types, and represents a promising pharmacological target.

2.3 Aims and outline of this study

Drugs aimed at regulating the adenosinergic system have been identified as promising therapeutic candidates with potential as inflammatory regulators. Medicinal chemistry has produced a large number of compounds capable of selectively binding the adenosine receptors (a selection of which is presented in table 1). However, our understanding about the underlying signaling pathways and the cellular effector mechanisms dictating adenosine's role in inflammation perpetuation or resolution has lagged behind, what has prevented the translation of promising candidate molecules (i.e. from disease models) into meaningful therapies. For this reason, the further development of AR-based strategies of immunomodulation requires knowledge of the molecular mechanisms that mediating adenosine's effects in inflammatory processes.

The aim of this work was to identify and characterize the cellular signals triggered by the activation of alternate ARs. A central aspect of adenosine's role in inflammation is the interplay between different signaling cascades activated by the different AR subtypes, which in turn determine the shutdown or the perpetuation of inflammation. Our work was focused on mast cells, a key cellular type in the initiation and maintenance of inflammatory responses. Mast cells are strategically located at the host-environment interface, and upon activation by intruder pathogens and derived molecules they can release a large number of mediators that act locally and that also initiate cell recruitment and thereby acquired immune responses. Beyond their function in initiating allergic responses through the release of pre-formed vasoactive amines and other immediate inflammatory mediators though degranulation, mast cells can influence the function of neutrophils, monocytes and even lymphocytes through *de novo* synthesis of cytokines and chemokines, and thereby influence the direction of the immune response being the actual effector cells. The LAD-2 cell line (Laboratory of Allergic Diseases-2) has more recently been established [171]. These resemble primary cultures of $CD34^{+}$-derived human mast cells, and are considered to be a well-differentiated mast cell line. Based on this, HMC-1 and LAD-2 cells have been employed as an in vitro surrogate model to study molecular processes associated to adenosine signaling.

In the first part of this thesis, we investigated A_{2A}AR signaling translated into inflammatory downregulation, in the human mast cell line HMC-1, which expresses A_{2A}, A_{2B} and A_3AR, and although it is an immature cell line, they have are suitable for the study degranulation independent process [172]. Transcriptional re-programming of HMC-1 cells upon treatment with a non-selective AR agonist (adenosine analogue) was compared with the pattern obtained by selective A_{2A}AR activation (A_{2A}AR agonist) and by those of non-A_{2A}AR mediated response (adenosine analogue plus an A_{2A}AR antagonist). We reasoned that genome-wide transcriptional screening would allow the identification of AR-specific downstream factors involved in the modulation of inflammatory signaling in mast cells. Furthermore, a sharp induction of transcriptionally active NR4A2 and NR4A3 by the adenosine analogue

NECA was observed. These results were confirmed by RT-PCR and western blot at protein level. Furthermore, through the combination of receptor assays and pharmacological inhibition of signaling kinase we established a previously unknown link between AR activation and nuclear orphan receptors NR4A2 and 3.

In the second part of this work the focus of our investigation was regulation of NR4As. The combination of PMA/ionomycin was employed to activate LAD-2 mast cell line. This cell line resembles the general characteristics and functions of primary tissue mast cells more closely than other cell lines. In particular, we wanted to determine whether modulation of the other pro-inflammatory signaling pathways by the A_{2A}AR plays a significant role in suppressing inflammation. In addition, it was also necessary to study the post-translational modification of NR4A and its translocation. Moreover, we would like to identify the new downstream targets and interaction partners of NR4A2. This was to be achieved by identifying stimuli that induce NR4A2 expression and A_{2A}AR could have consequences for regulating interactions between the A_{2A}AR and NR4A2.

3

Selective regulation of nuclear orphan receptors 4A by adenosine receptor subtype in human mast cell

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RESEARCH ARTICLE

Selective regulation of nuclear orphan receptors 4A by adenosine receptor subtypes in human mast cells

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Abstract Nuclear orphan receptors 4A (NR4A) are early responsive genes that belong to the superfamily of hormone receptors and comprise NR4A1, NR4A2 and NR4A3. They have been associated to transcriptional activation of multiple genes involved in inflammation, apoptosis and cell cycle control. Here, we establish a link between NR4As and adenosine, a paradoxical inflammatory molecule that can contribute to persistence of inflammation or mediate inflammatory shutdown. Transcriptomics screening of the human mast cell-line HMC-1 revealed a sharp induction of transcriptionally active NR4A2 and NR4A3 by the adenosine analogue NECA. The concomitant treatment of NECA and the adenosine receptor A_{2A} (A_{2A}AR) selective antagonist SCH-58261 exaggerated this effect, suggesting that upregulation of these factors in mast cells is mediated by other AR subtypes (A_{2B} and A_3) and that A_{2A}AR activation counteracts NR4A2 and NR4A3 induction. In agreement with this, A_{2A}AR-silencing amplified NR4A induction by NECA. Interestingly, a similar A_{2A}AR modulatory effect was observed on ERK1/2 phosphorylation because A_{2A}AR blockage exacerbated NECA-mediated phosphorylation of ERK1/2. In addition, PKC or MEK1/2 inhibition prevented ERK1/2 phosphorylation and antagonized AR-mediated induction of NR4A2 and NR4A3, suggesting the involvement of these kinases in AR to NR4A signaling. Finally, we observed that selective A_{2A}AR activation with CGS-21680 blocked PMA-induced ERK1/2 phosphorylation and modulated the overexpression of functional nuclear orphan receptors 4A. Taken together, these results establish a novel PKC/ERK/nuclear orphan receptors 4A axis for adenosinergic signaling in mast cells, which can be modulated by A_{2A}AR activation, not only in the context of adenosine but of other mast cell activating stimuli as well.

Keywords Nuclear orphan receptor 4A · Mast cells · Adenosine · Adenosine receptor

Abbreviations

AR	Adenosine receptor
HMC-1	Human mast cell line 1
NBRE-LUC	Nerve-growth factor I-B responsive element-luciferase reporter gene
NR4A1	Nuclear orphan receptor 4 A1
NR4A2	Nuclear orphan receptor 4 A2
NR4A3	Nuclear orphan receptor 4 A3
PKC	Protein kinase C
RT-PCR	Reverse transcriptase polymerase chain reaction

Concise summary Non-selective AR engagement triggers robust induction NR4A orphan receptors in mast cells. Induction of these early responsive genes can be blocked by selective activation of the anti-inflammatory A_{2A}AR not only in the context of adenosine but also of PMA signaling, suggesting a general mechanism of mast cell regulation.

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Introduction

NR4A orphan receptors are transcription factors that belong to the superfamily of steroid nuclear hormone receptors that have been associated with different cellular processes, including inflammation (Murphy et al. 2001; Pei et al. 2005), steroidogenesis (Manna et al. 2002), apoptosis (Winoto and Littman 2002), development of dopaminergic

neurons (Zetterstrom et al. 1997) and glucose metabolism (Pei et al. 2006b). This subfamily of nuclear orphan receptors is constituted by 3 members: NR4A1 (also known as Nur77 or nerve growth factor inducible B, NGFI-B), NR4A2 (also known as Nurr1) and NR4A3 (also known as NOR1). In the context of inflammation, these factors represent NF-κB and LPS-inducible genes in macrophages (Pei et al. 2005) and NR4A1 expression leads to induction of inflammatory genes, potentiating NF-κB pro-inflammatory signaling by IKKi kinase upregulation (Pei et al. 2006a). In addition, NR4A2 has been recognized as an important inducible factor in inflamed synovium and as a target of anti-inflammatory effects of methotrexate (Ralph et al. 2005).

Adenosine is a purine nucleoside normally present in the nanomolar range but its concentration in the extracellular space rises with increased oxygen consumption during hypoxia, tissue injury and inflammation. The rapid accumulation of adenosine is followed by biological responses through activation of 4 types of G-coupled adenosine receptors (ARs): A_1, A_{2A}, A_{2B} and A_3. Each of the receptor subtypes has a different pharmacological profile, tissue distribution and effector coupling profile. During chronic inflammatory processes the sustained formation of adenosine has been associated with deleterious effects. Elevated adenosine concentrations can be found, for example, in bronchoalveolar lavage and exhaled breath condesate of human patients with asthma (Driver et al. 1993; Huszar et al. 2002) where it perpetuates inflammation and contributes to airway hyperresponsiveness. However, activation of the A_{2A}AR has been associated to the suppression of inflammation and tissue remodelling (Fozard 2003; Ohta and Sitkovsky 2001) and inhibition of histamine and tryptase release from human mast cells (Suzuki et al. 1998). However, the transcriptional effectors downstream of AR activation responsible for adenosine's pro or anti-inflammatory effects remain to be characterized.

In this study, we employed the human mast cell HMC-1 to characterize subtype specific effectors of AR activation and we established a link between adenosine receptor activation and nuclear orphan receptors. Furthermore, we determined that activation of the A_{2A}AR counterbalances the induction of these transcription factors and that this effect is not limited to adenosinergic signaling but that it is also conserved for other mast cell activating stimuli. These observations describe a novel regulatory mechanism by A_{2A}AR, with implications in the progression of inflammation and related pathologies.

Materials and methods

Reagents and cell culture

All chemicals were obtained from Sigma-Aldrich (Switzerland) unless otherwise indicated. The human mast cell line-1 (HMC-1) was a kind gift from Dr. J. H. Butterfield, Mayo Clinic, Rochester, MN, USA and was grown in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen) supplemented with 10% iron supplemented fetal bovine serum (Invitrogen), 1.2 mM alphathiglycerol (Sigma) and 100'000 U/l Penicillin and 100 mg/l Streptomycin (complete IMDM) at 37°C in 5% CO_2.

Cell treatments

HMC-1 cells were seeded at 8×10^5 cells/ml and allowed to settle overnight. They were then treated at the indicated concentrations with the following chemicals: the adenosine analogue NECA (5′-N-Ethylcarboxamido-adenosine), the A_{2A}AR agonist CGS-21680 (9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine), the A_{2A}AR antagonist SCH-58261 (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine) and PMA (phorbol 12-myristate 13-acetate). The length of treatment varied between 30 min and 72 h. Cells were pretreated with 1 U/ml adenosine deaminase (Roche) to remove any pre-existing endogenous adenosine for 20 min.

RNA extraction and cDNA synthesis

Cells were collected by centrifugation at the corresponding time points and total RNA was recovered using the Qiashredder and Rneasy mini kit (Qiagen). Concentration and quality of total RNA were measured with the Ultraspec 2100 pro spectrophotometer (Amersham Biosciences). Samples with a UV absorbance 260/280 ratio of 1.8–2.1 were considered to be suitable for cDNA synthesis. RNA samples were stored at −20°C until use. Complementary DNA (cDNA) was synthesized using the one-cycle cDNA synthesis kit followed by a sample cleanup to optimize volumes and concentration of the cDNA (GeneChip sample cleanup module, Affymetrix).

Genome wide gene expression analysis

Global gene expression analysis was done in 4 independent experiments, consisting of the treatment with 10 μM NECA and untreated cells as a baseline control. After 3 h treatment of HMC-1 cells total RNA was extracted as described in the previous section. The quality of RNA was determined on the Agilent Lab-on-a-chip Bioanalyzer 2000 (Palo Alto, USA). Samples with a total area under 28S and 18S bands of less than 65% of total RNA, as well as a 28S/18S ratio of less than 1.5, were considered to be degraded and therefore excluded from microarray analysis. cRNA was synthesized from cDNA with the IVT labeling kit (Affymetrix). cRNA quality was assessed with the Agilent Lab-on-a-chip

Bioanalyzer 2000. The biotin-labeled cRNA was fragmented and hybridized on Human Genome U122 plus 2.0 microarrays (Affymetrix), which cover sequences of 47'000 human transcripts, following the manufacturer's instructions. After hybridization periods of 16 h the microarrays were automatically washed and stained on the Affymetrix Fluidics Station 450. Staining of the hybridized probes was performed with fluorescent streptavidin-phycoerythrin conjugates (1 mg/ml; Molecular Probes). The subsequent scanning of DNA microarrays was carried out on an Affymetrix scanner 3000 7 G. The generated data was then normalized and subsequently filtered using a significance value of $P< 0.05$ using the GeneSpring 7.3.1 sofware (Agilent, Palo Alto, CA, USA). We employed pair-wise analysis based on B - Fabric infrastructure tool (Functional Genomics Center Zurich, University of Zurich-Irchel, Switzerland) and the GeneGo Metacore (www.genego.com) integrated software for data mining and functional analysis of experimental data. Hierarchical clustering was performed with gplots library from CRAN.

Real time polymerase chain reaction (RT-PCR)

Specific primers for the selected transcripts as well as TaqMan probes and TaqMan master mix were obtained from Applied Biosystems. 40 ng of cDNA were mixed with 1 μl of forward and reverse primers and 10 μl of master mix supplemented with 25 nM of the corresponding TaqMan probe in a final volume of 20 μl. The reactions were performed in a 7500 Fast Real-time PCR-System ABI 7500 (Applied Biosystems) in 40 cycles (95°C for 3 s, 60°C for 30 s) after an initial 20 s incubation at 95°C, and was analyzed with the 7500 Fast System SDS Software System (Applied Biosystems). The fold change in expression of each gene was calculated with the 2-Delta Delta C(T) method. Each of these values had been normalized to the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blot

Thirty microgram of total protein in 1× Laemmli buffer were separated on a 10% polyacrylamide gel by standard SDS-PAGE technique from each sample, followed by transfer onto Immune-Blot polyvinylidene difluoride (PVDF) membranes (0.2 μm pore size, Bio-Rad) and blocking 16 h at 4°C with blocking solution (5% non-fat dry milk, 3% BSA in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4). NR4A2 and total ERK protein levels were assessed employing a monoclonal antibody (Alpha Diagnostic and R&D, respectively) and phosphorylated ERK1/2 by a specific T185/Y187 phopsho-antibody (R&D, USA). GAPDH and β-actin antibodies (Ambion, USA) were employed as internal loading controls. Enhanced chemiluminescence was performed with SuperSignal West Femto Maximum (Thermo Fisher Scientific, Switzerland) and images were acquired on a LAS-3000 image reader (Fujifilm Life Science, Japan). NR4A2 induction was quantified by calculating the ratio of intensity signals to GAPDH (Quantity 1 software, BioRad) and compared to untreated controls (value=1).

siRNA silencing of $A_{2A}AR$

0.4×10^6 HMC-1 cells in 1 ml were seeded in 24 well-plates. Cells were transcfected with 20nM $A_{2A}AR$ siRNA (Santa Cruz, Cat. sc-39850) using 4 μl lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions in Opti-MEM serum and antibiotics-free medium. After 5 h of incubation, the medium was replaced with complete medium. As a scramble negative control, control (FITC Conjugate)-A siRNA (Santa Cruz, sc-36869) was employed. Silencing of ARs was tested by RT-PCR with specific TaqMan probes (Applied Biosystems) as indicated above, and was maximal at 36 h. Therefore, this time point was selected for further analysis of AR signaling.

Transient transfection and luciferase reporter gene assay

1.5×10^6 HMC-1 cells in 1 ml were co-transfected with the tk-NBREx3-luc plasmid, which was a kind gift Dr. R Evans (Howard Hughes Medical Institute, San Diego, CA) and the pRL-TK vector (Promega, USA) at a ratio 30:1 in serum- and antibiotics-free Iscove's medium containing 8 μl of Lipofectamine 2000 reagent (Invitrogen). After 5 h of incubation, the medium was replaced with complete medium and were allowed to recover at 37°C for 24 h and were subsequently stimulated as indicated. For luciferase activity assay, cell lysates were prepared and assayed using the Luciferase Assay System (Promega, USA), according to the manufacturer's instructions. All luminescent measurements were performed automatically in a 96 well-plate in a Luminescence Spectrometer (MLX, Dynex).

Data analysis

Statistical analysis was performed using the PASW Statistics software (SPSS Inc., USA). Data were analyzed by nonparametric tests (Mann-Whitney U-test) and differences between groups were considered significant when p-values were less than 0.05.

Results

Transcriptional profiling of human mast cells reveals upregulation of NR4A family memebers by adenosine receptor activation

The human mast cell line HMC-1 exhibits a phenotype, which in several aspects is similar to tissue resident mast cells (Nilsson et al. 1994). However, these cells do not degranulate and therefore represent a suitable system for studying degranulation-independent de novo synthesis of inflammatory mediators.

Previous studies have successfully used this cell line to analyze adenosine signaling on human mast cells (Ryzhov et al. 2006; Ryzhov et al. 2004), which prompted us to employ a systematic genome-wide approach for the identification of novel effector molecules involved in AR signaling. For this, we stimulated HMC-1 cells to the adenosine analogue NECA and 3 h after exposure the total fraction of RNA transcripts was extracted, processed and hybridized to Affymetrix microarrays (4 replicates). To identify differentially expressed genes, the data sets of each replicate were analysed in a pair-wise fashion with the untreated controls as described in the methods section. This analysis yielded 19 NECA-induced transcripts with fold changes of three or higher (Table 1). The resulting heat map reflects the degree of similarity between the individual replicates (vertical dendogram, Fig. 1a). Gene ontology analysis of these transcripts revealed that most of these transcripts encode protein products involved in transcription regulation (NR4A2, NR4A3, CREM , EGR1, 2 and 3, FOSB, ELL2, ZEB1), signal transduction (HOMER1, ITK, IL2RB), hormone activity (STC1, CGA), DNA metabolism and repair (JMY, TP53INP2 ITK), cell cycle regulation (RGC32), apoptosis (BCL2L11) and tissue remodeling (PLAUR). Remarkably, two nuclear orphan receptors (NR4A2 and NR4A3), which had not been associated to adenosine signaling before, were among the highest upregulated genes. Interestingly, the third member of orphan receptor family, NR4A1, was induced only marginally (2.05 fold). These results were confirmed by real time RT-PCR (Fig. 1b) and suggest that NR4A2 and NR4A3 could mediate proinflammatory signaling by adenosine in mast cells.

A_{2A}AR inactivation amplifies NECA-mediated induction of NR4A2 and NR4A3

Human mast cells express A_{2A}, A_{2B}, and A_3ARs (Feoktistov and Biaggioni 1998; Feoktistov et al. 2003). Therefore, the observed response to NECA reflects the composite effect of activation of all 3 receptor subtypes. Because AR subtypes can promote (A_{2B} and A_3ARs) or downregulate (A_{2A}AR) inflammation, we wanted to determine the influence of A_{2A}AR in NR4A induction. To address this issue cells were stimulated either with NECA, with the selective A_{2A}AR agonist CGS-21680, or with a combination of NECA plus the A_{2A}AR antagonist SCH-58261, and NR4A induction

Table 1 Top regulated transcripts by non-selective AR stimulation. Cells were treated with 10 μM NECA and fold changes (FC) calculated against untreated control as described (fold change cut-off=3, $P<0.005$)

Gene Name	Description	NECA FC
NR4A2	Nuclear receptor subfamily 4, group A, member 2	33.52
STC1	Stanniocalcin 1	25.37
NR4A3	Nuclear receptor subfamily 4, group A, member 3	24.13
CREM	CAMP responsive element modulator	13.57
EGR3	Early growth response 3	10.16
RGC32	Response gene to complement 32	9.54
HOMER1	Homer homolog 1 (Drosophila)	7.95
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	6.91
CGA	Glycoprotein hormones, alpha polypeptide	6.14
ELL2	Elongation factor, RNA polymerase II, 2	6.14
JMY	Junction-mediating and regulatory protein	5.96
ZEB1	Transcription factor 8	5.42
EGR1	Early growth response 1	5.07
BCL2L11	BCL2-like 11 (apoptosis facilitator)	4.99
ITK	IL2-inducible T-cell kinase	4.76
PLAUR	Plasminogen activator, urokinase receptor	4.58
TP53INP2	p53 induced nuclear protein 2	3.88
IL2RB	Interleukin 2 receptor, beta	3.78
EGR2	Early growth response 2 (Krox-20 homolog, Drosophila)	3.72

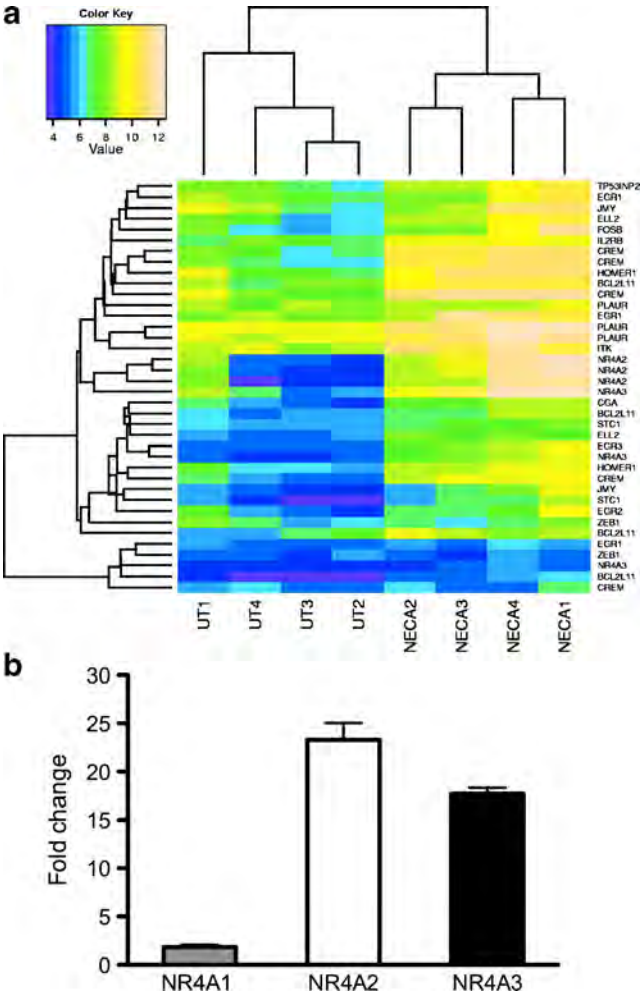

Fig. 1 **a** Hierarchical clustering analysis of transcriptional changes of HMC-1 cells induced by NECA. Each column represents a single experiment (4 replicates for each condition, untreated and treated with 10 μMNECA, 3 h) and contains all genes that were significantly upregulated in at least one of replicates and a minimum induction of 3 fold. The resulting vertical dendrogram indicates the degree of similarity between different transcriptomes. Expression levels are expressed in a log 2 scale and transcripts with values of 5 or lower (blue and light blue) are marginally expressed or absent. Repeated gene names indicate different probe sets for a single gene (different expression signals for alternative probe sets could indicate alternative splicing or poor probe performance). **b** Confirmation of NR4A induction by RT-PCR. Fold changes are calculated in relation to untreated controls. Values represent the mean of three experiments ± SEM

was assessed by RT-PCR. Figure 2a and b show time-dependent induction of NR4A2 and NR4A3. While NECA-mediated induction of NR4A2 and NR4A3 reached maximal induction of 240-fold and 98-fold respectively 1 h after treatment, selective activation of A_{2A}AR with 1 μM CGS-21680 did not induce them. In contrast, blockage of A_{2A}AR with the combined treatment of NECA and SCH-28261, results in further upregulation of these nuclear receptors, with fold changes up to 429 and 376 over the untreated controls respectively. These observations point out that the induction of these two transcription factors rely on the activation of *non-A_{2A} ARs*. Interestingly, NECA plus SCH-58261 did not upregulate NR4A1 further when compared to NECA (4.2-fold, Fig. 2c), indicating that this interplay between non-selective and selective A_{2A}AR activation is not conserved to the third member of this family of receptors. Also, induction of the cAMP-response element modulator (CREM, a top-regulated gene from the transcriptomics screening, see Table 1) reaches 55-fold when treated with NECA, while CGS-21680 or the combination of NECA plus SCH-58261 induce CREM only marginally, indicating that simultaneous activation of all ARs is required to achieve maximal CREM induction (Fig. 2d).

To confirm the role of A_{2A}AR dependent changes in NR4A2 and NR4A3 expression, HMC-1 cells were transfected with A_{2A}AR-specific siRNA, which led to 63% silencing 36 h after transfection (Fig. 3a). Treatment of A_{2A}AR silenced cells with NECA resulted in higher NR4A2 expression levels as compared to mock silenced cells, mimicking the effect of pharmacological blockage of A_{2A}AR with the combination of NECA plus SCH-58261 (Fig. 3b). Altogether, these results indicate that activation of A_{2A}AR counterbalances NR4A2 and NR4A3 induction by A_{2B}AR and A_3AR.

Adenosine receptor activation increases NR4A2 protein abundance

Next, we wanted to determine whether AR-mediated increment of NR4A2 mRNA levels translate into changes in protein abundance. Western blot analysis of NR4A2 protein revealed that NECA treatment results in a remarkable induction of this factors reaching maximal levels at 12 h, with values returning to background levels 24 h after exposure (Fig. 4). As expected, selective A_{2A}AR activation with CGS-21680 did not upregulate NR4A2 (not shown). However, induction of NR4A2 protein by the concomitant treatment of NECA plus SCH-58261 was higher than with NECA but also returned to basal levels within 24 h. These results confirm that transcriptional upregulation of NR4A2 by AR activation results in increased protein levels.

AR-induced NR4As are transcriptionally active

In view of the strong induction of NR4A2 and NR4A3, we performed a functional assay based on an exogenous reporter gene to determine how the activity of these factors is affected by AR activation. NR4A receptors bind to the octanucleotide 5′-A/TAAAGGTCA (NGFI-B response element, NBRE) and therefore we employed an NBRE-luciferase reporter plasmid (tk-NBREx3-luc) and quantified luciferase induction upon AR activation. Figure 5 shows

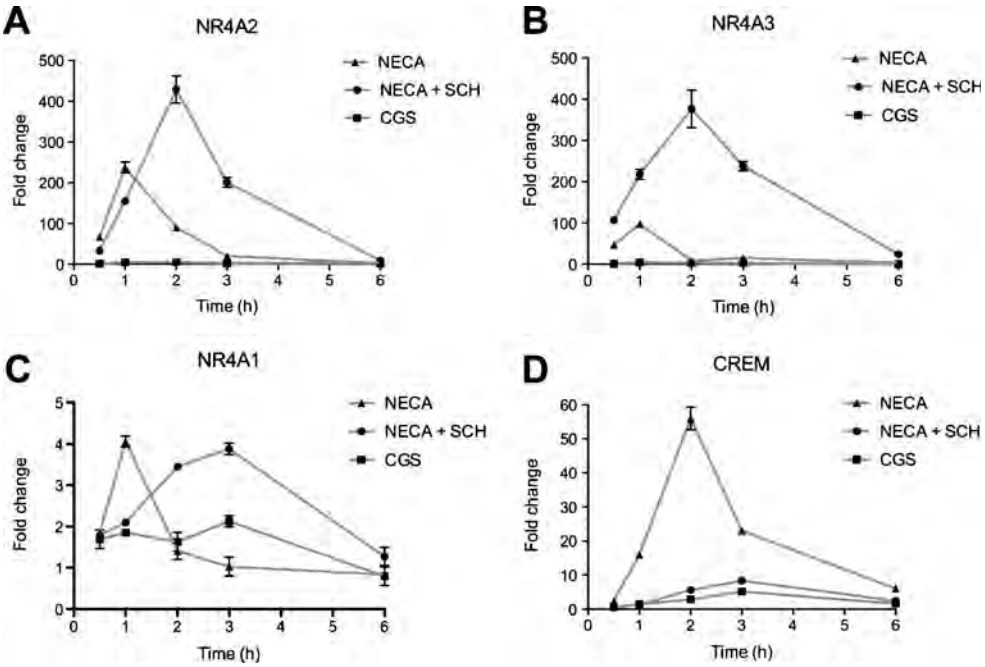

Fig. 2 Time dependent induction of NR4A2 (**a**), NR4A3 (**b**), NR4A1 (**c**) and CREM (**d**). Cells were treated with 10 μM NECA, 1 μM CGS-21680 (CGS) or 10 μM NECA plus 1 μM SCH-58261 (SCH) and RNA was collected at the indicated time points and analyzed by RT-PCR. Fold changes are calculated against untreated controls. Values represent the mean of three independent experiments ±SEM

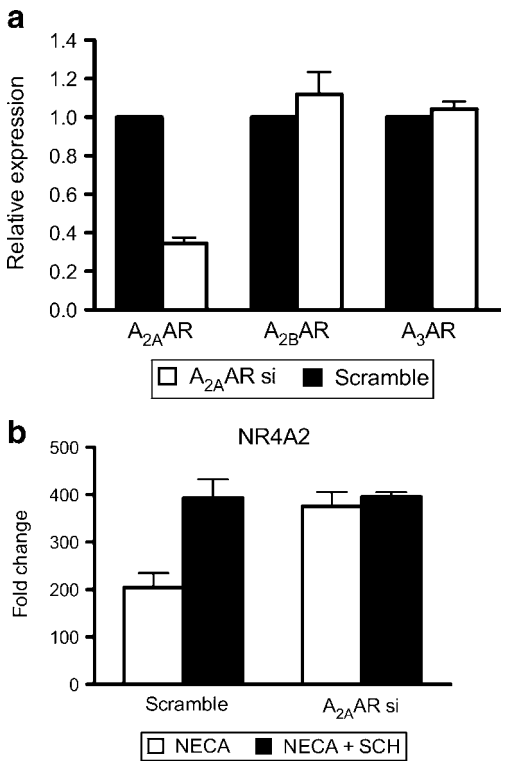

Fig. 3 Effect of $A_{2A}AR$ silencing on NR4A2 induction. **a** Transfection of HMC-1 cells with $A_{2A}AR$-siRNA ($A_{2A}AR$ si) specifically downregules this receptor subtypeation of $A_{2A}AR$. Scramble siRNA was included as a control **b** $A_{2A}AR$ silenced cells were treated with for 2 h with 10 μM of NECA or 10 μM of NECA plus 1 μM SCH-58261 (SCH). Values represent the mean of three independent experiments ± SEM

time-dependent induction of luciferase activity upon treatment with NECA, CGS-21680 or NECA in combination with SCH-58261. Both NECA and NECA plus SCH-58261 induced a robust response, which peaked between 12 h and 24 h with nearly a 30- and 40-fold reporter induction as compared to untreated controls respectively. NBRE reporter activity decreased sharply by 48 h and returned to background values 72 h after treatment. On the contrary, CGS-21680, did not induce luciferase expression significantly. Interestingly, reporter induction by NECA plus SCH-58261 was not statistically significantly higher than by NECA, despite the remarkable difference in induction of these transcription factors. This discrepancy can be explained by the intrinsic limitations of the artificial reporter gene system employed. The luciferase construct includes a minimal promoter region in the 5′-regulatory

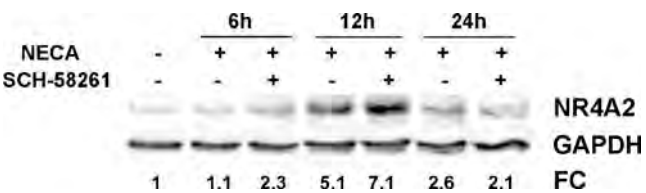

Fig. 4 Western blot analysis of NR4A2. HMC-1 cells were treated with 10 μM NECA, or the combination of 10 μM NECA and 1 μM SCH-58261 (SCH) for the indicated time points. GAPDH is included as an internal control. Fold change of NR4A2 protein (FC) are calculated from the ratio to GAPDH and compared to baseline expression in untreated cells (lane 1)

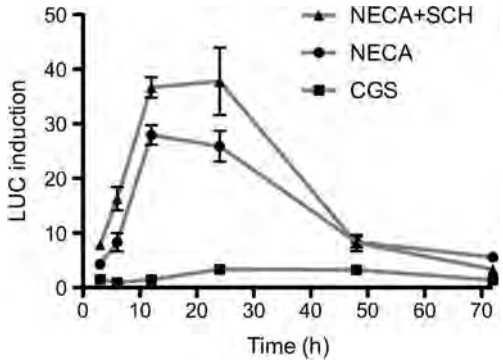

Fig. 5 Time-dependent induction of NBRE by AR engagement. The results are shown as ratio against the respective untreated control. For each measurement the ratio between the reporter gene (firefly) between and the internal Renilla standard was calculated (three independent determinations). Cells were treated with 10 μM NECA, 1 μM CGS-21680 (CGS) or 10 μM NECA plus 1 μM SCH-58261 (SCH)

region of the NBRE repeats. Therefore, this assay may not allow the full response (gene expression) that could be expected in the context of endogenous chromatin to take place, thereby limiting the amplitude of the responses measured and of the differences between treatments. Nevertheless, these results confirm that AR activation induces transcriptionally active NR4As that could have profound biological implications.

AR-mediated NR4A2 and NR4A3 upregulation involves PKC and MEK kinases, and correlates with A_{2A}AR-, PKC- and MEK-sensitive ERK1/2 phosphorylation

NR4As can be induced by a variety of stimuli, which are signaled through diverse intracellular regulators in a cell type and stimulus-dependent fashion (Martinez-Gonzalez and Badimon 2005). Therefore, we wanted to determine the intracellular signaling pathways participating in NR4A2 and NR4A3 induction by ARs. To address this issue we interrogated the role of the mitogen-activated kinase p38, protein kinase A (PKA), phosphoinositide 3 kinase (PI3K), protein kinase C (PKC) and MEK kinase with selective inhibitors, by assessing the AR-mediated induction of NR4A2 and NR4A3 (NECA plus SCH-58261). While 30 min pretreatment with SB203580 (p38 inhibitor), H89 (PKA inhibitor) and wortmannin (PI3K inhibitor) did not affect NR4A induction, blockage of either PKC or MEK with GF109203X or PD98059 respectively partially reverted AR-mediated induction of these factors (Fig. 6a). Based on previous reports that showed that ERK phosphorylation is involved in AR inflammatory signaling in HMC-1 cells (Feoktistov et al. 1999), and because ERK1/2 is a downstream target of PKC, we wanted to determine whether activation of A_{2A}AR correlates with ERK phos-

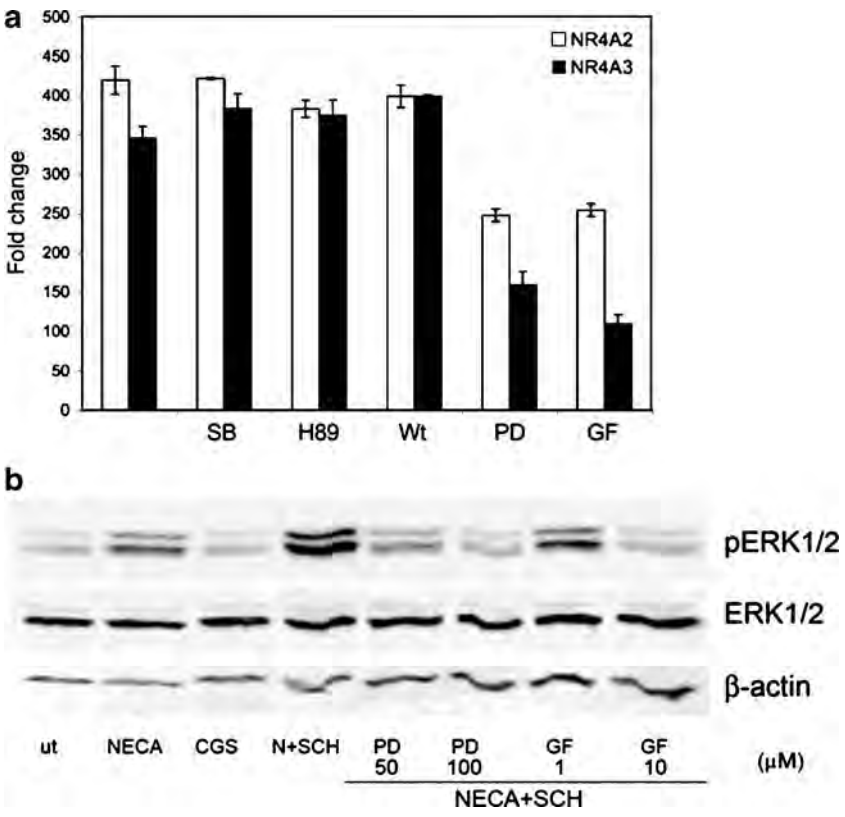

Fig. 6 Effect of kinase inhibitors on AR-mediated NR4A induction and ERK1/2 activation **a** Pretreatment with MEK and PKC inhibitors partially reversed NR4A2 and NR4A3 induction by 10 μM NECA and 1 μM SCH-58261. 0.5 μM SB203580 (SB), 1 μMH89, 0.1 μM wortmannin (Wt), 100 μM PD98059 (PD) or 10 μM GF109203X (GF) were employed for preincubation. **b** A_{2A}AR-blockage excacerbates NECA-induced phosphorylation of ERK1/2 (N+SCH). This effect is blocked by preincubation with PD98059 (PD) and GF109203X (GF) in a concentration dependent way

phorylation. NECA readily induced phosphorylation of ERK1/2 while CGS-21680 did not (Fig. 6b, lanes 2 and 3). Interestingly, NECA plus SCH-58261 resulted in exaggerated ERK1/2 phosphorylation (lane 4), demonstrating an inverse relationship between A_{2A}AR activation status and ERK1/2 phosphorylation. Furthermore, this pattern of ERK phosphorylation could be reverted by preincubation with GF109203X or with PD98059 in a concentration dependent manner (lanes 5–8). Taken together, these results indicate that PKC and MEK kinases are required for AR-dependent ERK1/2 phosphorylation and NR4A2 and NR4A3 upregulation, and that activation of A_{2A}AR opposes ERK1/2 activation by other ARs.

A_{2A}AR activation modulates PMA-induced ERK phosphorylation, NR4A2 and NR4A3 induction and NBRE transcriptional activity

Next, we wanted to assess whether A_{2A}AR activation is able to influence NR4A induction by stimuli other than adenosine. Therefore, we selected PMA, a diester of phorbol that acts as a PKC activator, which is a central regulatory molecule with a role in cytokine production, arachidonic acid release and mast cell degranulation (Chang et al. 1997; Cho et al. 2004). Stimulation of HMC-1 cells with PMA resulted in robust ERK1/2 phosphorylation that could be reverted with increasing concentrations CGS-21680 (Fig. 7a). In addition, 1 μM CGS-21680 significantly decreased PMA-mediated NR4A2 and NR4A3 induction (by 35 and 53% respectively, Fig. 7b) and, moreover, levels of PMA-induced NR4A2 protein were also reduced (Fig. 7c). In light of these findings, we tested whether A_{2A}AR activation could affect the transcriptional activity of PMA-induced NR4As, by means of the NBRE-LUC reporter assay. Remarkably, preincubation with 1 μM CGS-21680 caused a significant reduction in PMA-induced NBRE-LUC activity (35%, Fig. 7d).

Altogether, these results show that the inhibitory effect of A_{2A}AR on nuclear orphan receptor 4A stimulation is conserved beyond adenosinergic inflammatory signaling and that activation of this receptor can regulate intracellular signaling by other inflammatory stimuli.

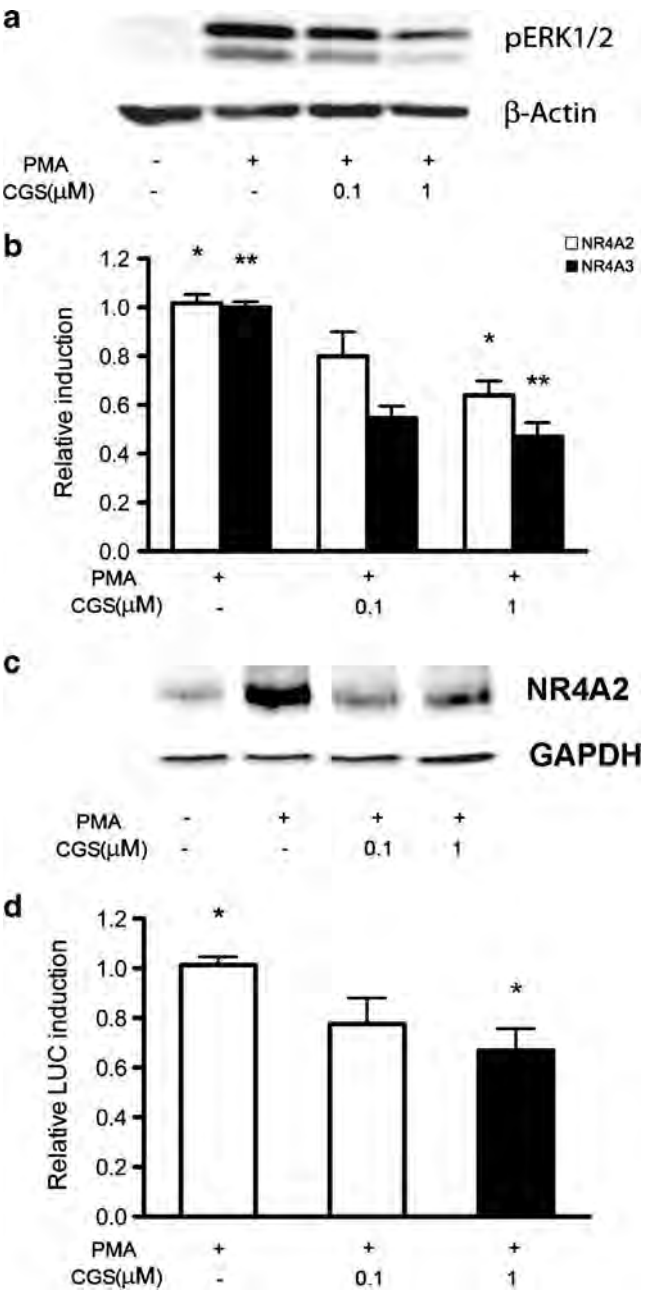

Fig. 7 Effect of A_{2A}AR activation on PMA-mediated NR4A activity. **a** Increasing concentrations of CGS-21680 (CGS) antagonize PMA-induced ERK1/2 phosphorylation. β-Actin is included as an internal control, **b** Reduction of PMA-mediated NR4A2 and NR4A3 transcriptional induction by increasing concentrations of CGS-21680 (CGS). Values are relative to induction by PMA and indicate the mean of three independent experiments ± SEM. *, **: $p<0.05$ for reduction in NR4A2 and NR4A3 induction respectively (Mann-Whitney U test), **c** Western blot analysis of NR4A2 induced by PMA alone or with the indicated concentrations of CGS-21680, **d** CGS-21680 (CGS) reduces the transcriptional activity of PMA-mediated NBRE-LUC reporter activity. For each measurement the ratio between the reporter gene (firefly) and the internal Renilla standard was calculated. Values are relative to LUC expression by PMA, and represent the mean of three independent experiments ± SEM. PMA concentration in all experiments was 50 nM. *$p<0.05$ for NBRE-LUC activity reduction (Mann-Whitney U test)

Discussion

Mast cells have been traditionally associated to immediate-type hypersensitivity reactions through the release of preformed inflammatory mediators. However, in recent years it has become clear that these cells can regulate immune responses through de novo synthesis of cytokines, chemokines, and eicosanoids without degranulation (differential release inflammatory mediators), supporting a role of

mast cells in more persistent (chronic) inflammatory and immunological responses such as chronic bronchitis and asthma-related pulmonary inflammation (Brightling et al. 2003; Church and Levi-Schaffer 1997), renal injury (Mack and Rosenkranz 2009), and tumorigenesis (Blatner et al. 2010; Groot Kormelink et al. 2009).

The engagement of activating and inhibitory cell-surface receptors, as well as the intensity and duration of these signals, will determine the activation state of mast cells; in response to these stimuli, gene expression patterns of inflammatory mediators are altered according to the sum of positive and negative signaling events, thereby affecting the course of inflammation. Based on adenosine's ability to both positively and negatively regulate mast cell activation through the engagement of alternate AR subtypes, it has been argued that selective activation of the A_{2A}AR (anti-inflammatory) or blockage of A_{2B}AR and A_3AR (proinflammatory) may represent a pharmacological tool for modulating their adenosinergic inflammatory signaling. However, transcriptional effectors downstream of ARs responsible for adenosine's pro- or anti-inflammatory effects remained to be fully characterized.

In this study we present a novel link between AR activation and NR4A orphan receptors in human mast cells, and we show that selective activation of the A_{2A}AR can negatively regulate the induction of these factors. Our initial genome-wide screening revealed strong upregulation of NR4A2 and NR4A3. Recent evidence shows that NR4A2 induction represents a common point of convergence of distinct cytokine signaling pathways, suggesting an important common role for this family of transcription factors as mediating inflammatory signaling (McEvoy et al. 2002). Therefore, we reasoned that the anti-inflammatory A_{2A}AR could influence the expression of these pro-inflammatory factors. In fact, we observed that the concomitant treatment with the A_{2A}AR antagonist SCH-58261 significantly amplified NECA's effect on NR4A2 and NR4A3 induction. In view of the pleiotropic physiological roles of NR4As, adenosine's effect on this group of transcription factors could have broad biological implications.

In contrast to other members of the nuclear hormone receptor superfamily, the crystal structure and NMR data indicate that the ligand-binding pocket of NR4A receptors is covered by hydrophobic residues (Wang et al. 2003). In fact, these receptors have been shown to function as ligand-independent transcription factors that are constitutively active and whose activity is controlled at the level of protein expression and post-translational modifications (Codina et al. 2004; Fahrner et al. 1990). For this reason, adenosine's effects on the abundance of these transcription factors could have immediate biological implications. On one hand, the activation of pro-inflammatory ARs (i.e. A_2B and A_3ARs) in inflammatory cells could act as an amplification signal resulting in even higher levels of NR4As and in the expression of a larger set of NBRE-responsive genes. On the other, A_{2A}AR activation could mediate inflammation resolution indirectly by limiting the expression of NR4A2 and NR4A3-dependent inflammatory genes.

NR4As also influence the function of other inflammation-associated transcription factors. For example, NR4A1 and NR4A2 form heterodimers with retinoic acid receptor and can influence retinoid signaling (Wallen-Mackenzie et al. 2003). Therefore, AR activation could affect the number of NR4A-containing complexes: AR-mediated accumulation of NR4A2 and NR4A3 would translate in a higher proportion of transcriptional complexes containing these orphan receptors. Conversely, A_{2A}AR activation would limit the availability of NR4A2 and NR4A3 for heterodimerization with other TFs. In addition, NR4A receptors can also crosstalk with other TFs and influence their activity without necessarily interacting with them. A recent study has established that NR4A receptors and the estrogen-related receptors NR3B mutually repress each others transcriptional activity (Lammi et al. 2007). Similarly, NR4A1 has been shown to negatively cross-talk with NF-κB (Harant and Lindley 2004). As a consequence, by virtue of the induction of reamarkably high levels of functional NR4A2 and NR4A3, adenosine is likely profoundly affect the expression of large sets of genes both directly (NBRE-responsive transcripts) and indirectly, by affecting the nature of transcriptional complexes and/or crosstalking with other TFs.

Activation of mast cells (for example by the high affinity IgE receptor) requires the activation of receptor-proximal tyrosine kinases, mobilization of internal Ca^{2+} and the formation of signaling complexes coordinated by adaptor proteins (Rivera and Gilfillan 2006). PI3K is a central player in mast cell activation that signals to regulatory molecules such as PKC and phospholipases C and D (PLC and PLD) among others, which ultimately regulate mast cell degranulation, arachidonic acid metabolite production and cytokine gene transcription. AR activation has traditionally been linked to stimulation or inhibition of the adenylyl cyclase; A_{2A}AR and A_{2B}AR activation lead to increased cAMP levels that in turn activate the canonical PKA pathway and the exchange protein directly activated by cAMP (Epac) (Palmer and Trevethick 2008), while A_1AR and A_3AR activation leads to cAMP decrease (Zhou et al. 1992). In addition, AR signaling in mast cells has also been linked to PLC and calcium mobilization (A_{2B}AR and A_3AR), PI3K (A_3AR), as well as PKC and MAP kinases (A_1, A_{2A} and A_3AR) (Jacobson and Gao 2006; Spicuzza et al. 2006).

Several intracellular regulators have been linked to NR4A induction (Martinez-Gonzalez and Badimon 2005). In this study we established that AR-mediated NR4A2 and NR4A3 upregulation in HMC-1 did not involve PKA nor

PI3K nor p38. Instead, PKC and MEK kinase inhibition could partially revert the induction of these factors and, moreover, the activity of these kinases correlated with ERK1/2 phosphorylation. Interestingly, some studies have also shown the involvement of ERKs in adenosine signaling (Feoktistov et al. 1999) but downstream targets of ERK upon AR activation had remained elusive. The results presented here suggest that activation of ERK1/2 kinases downstream of PKC mediates NR4As induction by AR. Remarkably, A_{2A}AR can counterbalance NECA-induced ERK1/2 phosphorylation, correlating with its modulatory effect on NR4A2 and NR4A3 induction. However, blockage of PKC and a complete inhibition of ERK phophorylation resulted only in about a 50% decrease NR4A2 and NR4A3 induction, suggesting the involvement of at least one additional intracellular signaling pathway in AR-dependent nuclear orphan receptor upregulation in mast cells. The contribution of other intracellular signaling pathways remains to be investigated.

Finally, we examined whether the antagonistic effect of A_{2A}AR on ERK1/2 phosphorylation and NR4A2 and NR4A3 induction is preserved to other mast cell activating stimuli, by the concomitant activation of A_{2A}AR and mast cell stimulation with PMA. Strikingly, A_{2A}AR engagement resulted not only in a modulation of PMA-mediated ERK1/2 phosphorylation, but also limited NR4A2 and NR4A3 induction, and their activity as assessed by the NBRE-LUC reporter assay. These results show that A_{2A}AR modulatory's effect on the ERK1/2-NR4A signaling axis is not limited to adenosinergic signaling.

Taken together, the results presented in this study establish a novel effector signaling axis downstream of adenosine, and suggest NR4A antagonism as a mechanism mediating A_{2A}AR anti-inflammatory effects in mast cells. Thus, this data contributes to the understanding of how receptor-specific signals are integrated towards modulation of the inflammatory response, which could facilitate the development of AR-based strategies of immunomodulation.

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Interplay between the nuclear orphan receptor NR4A2, NF-κB and the adenosine receptor A2A in the regulation of inflammatory IL-4 production in mast cells

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Interplay between the nuclear orphan receptor NR4A2, NF-κB and the adenosine receptor A2A in the regulation of inflammatory IL-4 production in mast cells

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Abstract

Nuclear orphan receptors 4A (NR4A) are early responsive genes that belong to the superfamily of hormone receptors and comprise NR4A1, NR4A2 and NR4A3. Here we report that the stimulation of LAD-2 human mast cells with a combination of phorbol 12-myristate 13-acetate PMA (P) and the calcium ionofore Ionomycin (I) led to NR4A2 upregulation. In addition, we describe that this treatment induces phosphorylation-dependent ubiquitination of NR4A2. Cell fractionation and immunofluorescence approaches showed that P/I-induced phosphorylation-dependent ubiquitination led to an accumulation of NR4A2 to the endolysosomes and endoplasmic reticulum 6 h after treatment, and that inhibition of the proteasome with MG132 increased the accumulation of modified NR4A2 in these organelles. To characterize the biological meaning of NR4A2 post-translational modifications we analysed changes in NR4A2 downstream effectors, in particular of IL-4. P/I treatment strongly induced IL-4 transcription and release, which was dependent on PKC/MEK/ERK signaling and could be reverted NR4A2 silencing. MG132 pretreatment enhanced P/I-induced IL-4 release 6 h after treatment (in correlation with the accumulation of NR4A2 in cytoplasmic organelles), but strongly reduced IL-4 release, despite the effect of MG132 on stabilizing NR4A2 in cytoplasmic organelles. These observations suggested the late release of IL-4 could depend on another factor. In fact, pretreatment with the NF-κB inhibitor BAY 11-7082 significantly inhibited the release of this cytokine at a late time point and MG132 also inhibited NF-κB activation, strongly suggesting that the late inhibitory effect of MG132 on IL-4 release could be mediated by inhibition of NF-κB signaling.

Furthermore, NR4A2 silencing partially reduced NF-κB-luc reporter activity, suggesting that NR4A2 is required for the activation NF-κB at a later time-point. These results indicate that while NR4A2 is the central transcriptional factor driving IL-4 release in the early phase (6 h), NF-κB becomes determinant factor in IL-4 synthesis later time points. Finally, selective A_{2A}AR activation (CGS-21680) showed the inhibitory effect on NR4A2 expression and NF-κB activity, as well as IL-4 induction. Thus, our data presented here suggests that through the modulation of IL-4 release via NR4A2 and NF-κB signaling, A_{2A}AR activation in mast cells can negatively influence the development of allergic Th2 responses.

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Introduction

The NR4A subfamily of orphan nuclear receptors include NR4A1 (Nur77), NR4A2 (Nurr1) and NR4A3 (NOR-1). NR4A receptors are rapidly and strongly induced by a wide array of stimuli, such as stress, growth factors and inflammatory signals [1-3], and they have been implicated as important transcriptional mediators of inflammatory signals in several organs including lung [4], brain [5], liver [6], skeletal muscle [7], blood vessels [8], adipose tissue [9] and immune cells [10]. NR4A2 has been recognized as an important inducible factor in inflamed synovium and as a target of anti-inflammatory effects of methotrexate [11].

Because they are constitutively active, ligand-independent transcription factors, the activity of NR4As is tightly controlled at the level of expression. In addition, NR4A1 has been shown to be modulated by phosphorylation modifications that shuttles this factor to the mitochondria acting as an apoptotic signal [12]. In the case of NR4A2, phosphorylation has been shown to affect the transcriptional activity of this factor [13]. However, although NR4A2 has been shown to possess putative sumoylation sites [14], no furhter post-translational modifications of this factor have been characterized to date. A recent study has shown a different expression and nucleocytoplasmic pattern for NR4A2, suggesting that yet undescribed post-translational modifications could control the cellular distribution of this factor [15] .

Adenosine is a purine nucleoside that accumulates in the extracellular space in response to increased oxygen consumption during hypoxia, tissue injury and inflammation [16]. This nucleoside acts as a rapid inflammatory signal, triggering divergent biological responses through the activation of 4 types of G-coupled adenosine receptors (ARs). While the ARs A_1 and A_{2B} and A_3 have been mainly associated with pro-inflammatory signaling [17-19], activation of the A_{2A}AR in a wide variety of immune cells results in a wide range of anti-inflammatory and immunosuppressive responses, including cytokine production, immune cell proliferation, leukocyte recruitment and phagocytosis [20-22] and the suppression tissue remodeling [23, 24].

The role of mast cells as effector cells in a variety of pathological conditions [25] is becoming better understood. Classically, mast cells have been associated with the release of preformed mediators stored in the secretory granules upon the engagement of high affinity IgE receptors (FcεRI) [26]. However, several other stimuli are capable of inducing upregulation of inflammatory cytokines independently of degranulation,

including inflammatory mediators, complement factors, and bacterial and viral components [27]. Recently, we established that activation of pro-inflammatory ARs induce a rapid and robust upregulation of NR4A2, which could be counterbalanced by the simultaneous activation of the anti-inflammatory A_{2A}AR receptor [1-3].

To further our understanding of the mechanisms involved in NR4A2 signaling in activated mast cells, in this study we characterized the sequence of events triggered by the concomitant activation with PMA and the calcium ionofore ionomycin in LAD-2 human mast cells. We we report that this treatment results in upregulation and phosphorylation-dependent ubiquitination of NR4A2 and that this post-translational modfication induces its accumulation in endoplasmic reticulum (ER), and the endo-lysosomal compartment, which correlated with the induction of ER-stress markers. Furthermore, this treatment results in a NR4A2-dependent sustained release of IL-4, although NF-κB is required for the late phase release of this interleukin.

Materials and methods

Reagents and cell culture

All chemicals were obtained from Sigma-Aldrich (Switzerland) unless otherwise indicated. LAD-2 cells were kindly provided by Dr. A. Kirshenbaum (Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, MD 20892-1881). Cells were incubated at 37 °C, 5% CO_2 in StemPro-34 serum-free medium (Invitrogen, Switzerland), supplemented with 2 mM glutamine, 100 IU/mL penicillin, 50 µg/mL streptomycin and 100 ng/mL recombinant human stem cell factor (Peprotech, UK). Cells were not grown above a density of 0.5×10^6 cell/ml.

Cell treatments

LAD-2 cells were seeded at 8×10^5 cells/ml and allowed to settle overnight. They were then treated at the indicated concentrations with the following chemicals: PMA (phorbol 12-myristate 13-acetate), ionomycin (calcium ionophore A23187), the adenosine analogue NECA (5′-N-Ethylcarboxamido-adenosine), ubiquitin E1 ligase inhibitor UBEI-41 (4 [4-(5-Nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester) (BioGenova, US), the A_{2A}AR agonist CGS-21680 (9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine) and the proteasome inhibitor MG132 (N-(benzyloxycarbonyl) leucinylleucinylleucinal Z-Leu-Leu-Leu-al).

RNA extraction and cDNA synthesis

Cells were collected by centrifugation and total RNA was recovered using the NucleoSpin RNA II (Macherey-Nagel, Switzerland). Concentration and quality of total RNA were measured with the Ultraspec 2100 pro spectrophotometer (Amersham Biosciences, Switzerland). Samples with a UV absorbance 260/280 ratio of 1.8–2.1 were considered to be suitable for cDNA synthesis. RNA samples were stored at −20°C until use. Complementary DNA (cDNA) was synthesized using high-capacity cDNA reverse transcription kits according to the manufacture’s protocols (Applied biosystem, Switzerland).

Real time polymerase chain reaction (RT-PCR) and RT-PCR human chemokine array

Specific RT-PCR primers for the selected transcripts as well as TaqMan probes and TaqMan master mix were obtained from Applied Biosystems. 40 ng of cDNA were mixed with 1 µl of forward and reverse primers and 10 µl of master mix supplemented with 25 nM of the corresponding TaqMan probe in a final volume of 20 µl. The reactions were performed in a 7500 Fast Real-time PCR-System ABI 7500 (Applied Biosystems, Switzerland) in 40 cycles (95°C for 3 s, 60°C for 30 s) after an initial 20 s incubation at 95°C. For RT-PCR array, cDNA (40 µg) was mixed with 500 µl Taqman master mix, and the final volume of 10 µl/well was loaded on 96 well human chemokine gene array, which was preloaded 96 Taqman probes in the plates (Applied Biosystems, Switzerland). Quantitative RT-PCR was run for 35 cycles in standard mode using an ABI 7500. Raw data were processed using SDS 2.2.1 software (Biorad, Switzerland). The fold change in expression of each gene was calculated with the 2-Delta C(T) method. Each of these values had been normalized to the control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

siRNA knockdown

LAD-2 cells were transcfected with 20 nM siNR4A2, siA_{2A}AR, siCHOP, siRELA (NF-κB p65) (Qiagen, Switzerland) using 4 µl lipofectamine RNAiMax (Invitrogen, Switzerland) according to the manufacturer's instructions in Opti-MEM (Invitrogen, Switzerland) serum and antibiotics-free medium. After 5 h of incubation, the medium was replaced with complete medium for 48 h. As a scramble negative control, control (FITC Conjugate)-A siRNA (Santa Cruz, Switzerland) was employed. Knockdown efficacy was tested by rt-PCR or western blot analysis.

Co-immunoprecipitation assay

LAD-2 cells were stimulated with PMA/ionomycin (P/I), and lysed 6 h in the 2x detergent lysis buffer containing 2% SDS, 100 mM Tris (pH 7.5), 10 mM DTT, Proteinase Inhibitor Cocktail (Roche, Switzerland). They were immediately boiled for 10 min and sonicated for 4 s. After centrifugation, the supernatant was diluted with 4 volumes of NP-40 buffer with containing 25 mM Tris (pH 7.5), 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 0.25 mM PMSF, 200 mM NaCl and Proteinase Inhibitor Cocktail. The mixture was incubated with anti-NR4A2 antibody or IgG protein

overnight at 4 °C. Complexes were covered by incubation with 30 µl of protein A agarose/Salmon Sperm DNA (Millipore, Switzerland) for 10 h at 4 °C. Precipitates were washed serially with 1 ml of TNET buffer twice (50 mM Tris, pH 7.5, 140 mM NaCl, 1% Triton X-100), and then twice with 1 ml of TNE buffer (50 mM Tris, pH 7.5, 140 mM NaCl). The proteins remaining with the resin were analysed by immunoblot assays using the anti-NR4A2 (Santa Cruz, Switzerland) and anti-Ubiquitin (FK-2, Millipore, Switzerland).

Subcellular fractionation

Briefly, LAD-2 cells were harvested and washed twice with PBS. Cell pellets were resuspended in fraction buffer which contained 250 nM sucrose, 20 nM HEPES, 10 mM KCL, 1.5 mM $MgCl_2$, 1 mM EDTA, and left on ice for 20 min. Spun it for 5 min at 720× *g*. The supernatant (cytoplasmic) was collected for further analysis. The pellet was washed once with fraction buffer and dispersed with a pipette and pass through a 25 G needle 10 times. Centrifuged again at 3,000 rpm for 10 min. Removed the wash buffer and resuspended the pellet (nuclei) in Laemmli buffer, sonicated briefly on once before further analysis. Mitochondria and endoplasmic reticulum (ER) fractions were isolated according to the published protocols [28]. Briefly, 4 x 10^7 LAD-2 cells were wash with PBS twice and resuspensed in homogenization buffer (0.25 M cucrose, 10 mM HEPES, pH 7.4). Crude ER (supernatant, SN) was separated form crude mitochondria (pellet) by centrifugation at 15,000 *g* for 10 min. For ER purification, SN was loaded to discontinuous sucrose gradients and centrifuged at 28,000 rpm for 70 min (AH-629 rotor, Sovall). Collected ER gradients and centrifuged at 26,000 rpm for 45 to pellet, and then resuspended ER pellet for further analysis. For mitochondrial purification, crude mitochondrial (pellet) was resuspended to sucrose gradients and centrifuged at 27,000 rpm for 22 min (TLA 100.4 rotor). Collected mitochondrial gradients and spun at 15,000 *g* for 10 min to get mitochondrial pellet. Lysosomal fraction was performed mainly as described by Kjeldsen et.al for neutrophils [29]. Briefly, 3x10^7 LAD-2 cells were washed once with 25 ml of phosphate buffered saline (PBS). The pellet was resuspended in 700 µl of homogenization buffer (HB; 340 mM sucrose, 10 mM HEPES, 0.3 mM EDTA, pH 7.3) and subjected to 2 freeze/thaw cycles. The homogenate was sonicated and then spun at 3,000 rpm for 10 min to pellet nuclei. The PNS was collected and applied on a two-layer Percoll gradient. The Percoll was diluted with HB and gradient densities

were 1.05 and 1.12 (1.2 ml/layer). The gradient was layered in 3.2 ml polycarbonate ultracentrifugation tubes (Beckman, Switzerland). After applying the PNS on top of the gradient, the samples were spun at 30,000 rpm for 50 min (TLA 100.4 rotor) in a Beckman Optima TLX ultracentrifuge (Beckman). Fractions of 100 µl were collected starting from the top of the gradient. The fractions were washed twice at 43,000 rpm for 45 min for removing percoll and stored for further analysis.

Western blot

30 µg of total protein in 1× Laemmli buffer was separated on a 10% polyacrylamide gel by standard SDS-PAGE technique from each sample, followed by transfer onto Immune-Blot polyvinylidene difluoride (PVDF) membranes (0.2 µm pore size, Bio-Rad, Switzerland) and blocking 16 h at 4°C with blocking solution (5% non-fat dry milk, 3% BSA in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4). NR4A2 protein levels were assessed employing the NR4A2 antibody (1:2,000), phosphorylated ERK1/2 (1:2,000) by a specific T185/Y187 phospho-antibody and quantified by total ERK1/2 (1:2,000) (R&D, UK. Anti-tubulin (1:5,000, Ambion, Switzerland), anti PARP-1 (1:2,000, Alexis, Switzerland), anti-MnSOD (1:2,000, BD biosciences, Switzerland), anti-PERK (1:2000, Cell signaling, Switzerland), anti-LAMP1 (1:2,000, Santa cruz) were using as the marker for cytoplasm, nuclear, mitochondrial, endoplasmic reticulum and lysosome, respectively. Anti-GAPDH antibody (1:10,000, Ambion) was employed as internal loading control. Enhanced chemiluminescence was performed with SuperSignal West Femto Maximum (Thermo Fisher Scientific, Switzerland) and images were acquired and quantified on a LAS-3000 image reader (Fujifilm Life Science, Japan). Protein induction was calculated the ratio of intensity signals to GAPDH (Quantity 1 software, BioRad) and normalized to untreated controls.

Immunofluorescence

$1x10^6$ LAD-2 cells were transfected with 2 µg eGFP-Rab5, eGFP-Rab7, eGFP-LAMP1 and eGFP control plasmids with 10 µl Lipofectamine 2000 (Invitrogen) for 4 h. After different stimulations, the cells were cytospun on the slides and fixed with 4% paraformaldehyde (PFA) in PBS for 10 min at room temperature (RT), permeabilized with 0.2% triton X-100 in PBS for 5 min at RT, blocked with 0.1% BSA in PBS for 30 min at 37°C, and incubated 1 h at 37°C with the anti-NR4A2

antibody (1：200, Santa cruz) or anti-calnexin antibody (1:200, Santa cruz). Next, coverslips were incubated for 1 h with AlexaFluor594-conjugated goat anti-rabbit secondary antibody (1:200, Invitrogen) or AlexaFluor488-conjugated goat anti-mouse secondary antibody (1:200, Invitrogen). For counterstaining, cells were incubated for 10 min at 37°C with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in PBS. Between each step, the sections were washed in PBS 3 times for 5 min each. After washing, coverslips were mounted on microscope slides with Fluoromount G (Interchim, Switzerland) and analyzed in a confocal microscope (SP5, Leica Microsystems, Germany), using an oil immersion 63× objective. Co-localization of staining was analyzed in single optical sections obtained for three channels by Imaris (Bitplane, Switzerland). The results were analyzed from cells expressing NR4A2/calnexin (n=32), NR4A2/eGFP-Rab5 (*n* = 35), NR4A2/eGFP-Rab7 (*n* = 36), and NR4A2/ eGFP-Lamp1 (*n* = 28). The intensity of foci was quantified by the function of 'Analysis Particles', and the colocalization ratio of NR4A2/endolysosome was analyzed by JACoP plugin of ImageJ and Imaris 'color' function.

IL-4 ELISA

The IL-4 level in the cell culture supernatants were determined with an anti-human IL-4 ELISA kit (Peprotech, UK) according to the manufacturer's instructions. Briefly, ELISA plates were coated with the capture antibody (0.5 μg/ml) by overnight incubation at RT. Nonspecific binding sites were blocked by 1 h incubation at RT with 1% BSA in PBS. The supernatants from the LAD-2 cultures were tested by 2 h incubation at RT. The plates were then incubated for 2 h at RT with the detection antibody (0.25 μg/ml), followed by 30 min incubation with avidin-HRP conjugate. Bound complexes were detected by reaction with ABTS liquid substrate. Absorbance was measured at 405 nm using an ELISA plate reader. The cytokine concentration in the samples was calculated as pg/ml using recombinant human IL-4 as a standard.

Transient transfection and luciferase reporter gene assay

1.5×10^6 LAD-2 cells in 1 ml were co-transfected with the tk-NBREx3-luc plasmid, which was a kind gift Dr. R Evans (Howard Hughes Medical Institute, San Diego, CA) or wt-κB and the pRL-TK vector (Promega, Switzerland) at a ratio 30:1 in Opi-TEM medium containing 10 μl of Lipofectamine 2000 reagent. After 5 h of

incubation, the medium was replaced with complete medium and were allowed to recover at 37°C for 24 h and were subsequently stimulated as indicated. For luciferase activity assay, cell lysates were prepared and assayed using the Luciferase Assay System (Promega), according to the manufacturer's instructions. All luminescent measurements were performed automatically in a 96 well-plate in a Luminescence Spectrometer (MLX, Dynex, Switzerland).

Results

PMA and ionomycin triggers NR4A2 upregulation and phosphorylation-dependent ubiquitination

Members of the NR4A subfamily of orphan receptors have emerged as key transcriptional regulators of cytokines that can be strongly induced by inflammatory stimuli. Here we wanted to establish the involvement of NR4A2 in PMA and ionomycin (P/I) signaling in LAD-2 mast cells. RT-PCR analysis showed that 6 h after treatment both PMA and Ionomycin induced a remarkable upregulation of NR4A2 in LAD-2 cells, and that the combined P/I treatment potentiated this effect (Fig 1A). In agreement with our previous results, NR4A2 induction with the adenosine analogue NECA at this time point was lower [1]. None of these treatments affected cell viability (supplementary Fig 1). Western blot analysis confirmed that NECA, PMA and Ionomycin treatment lead to increased NR4A2 protein levels (Fig 1B). Interestingly, in addition to the bands at the expected NR4A2 molecular weight (66 kDa), P/I resulted in additional slow migrating bands, with a predominant one of about 77KDa (Fig 1B). To confirm the identity of this band, LAD-2 cells were transfected either with NR4A2 specific or scramble siRNA (efficiency of NR4A2 silencing 74.2%, supplementary Fig 2) and analysed by western blot (Fig 2). This experiment confirmed the identity of these slow migrating bands, suggesting that P/I treatment induces post-translational modifications (PTM) on NR4A2.

The size difference between the expected (66KDa) and the main slow migrating band (77KDa) would suggest that NR4A2 had been modified by ubiquitination. To test this hypothesis, cell lysates lysates were incubated with anti-NR4A2 antibody, and the enriched fractions tested by co-imunoprecipitation with an anti-ubiquintin antibody (Fig 3A). High molecular weight smear-like signals were detected between the 75 and the 100KDa MW marker. In addition, treatment with the E1 ubiquitination inhibitor 41 (UBEI-41), attenuated the 77KDa NR4A2 band in a dose dependent manner (Fig 3B). In fact, UBEI-41 concentrations of 25 μM and higher completely reverted the shifted pattern, resulting in abundant unmodified NR4A2 at 66KDa.

Next, we wanted to determine whether ubiquitination is the primary PTM on NR4A2 in response to P/I, or whether ubiquitination depends on other PTM. The presence of much weaker high-molecular weight bands associated to the main 77KDa band, would be compatible with phosphorylation. Therefore LAD-2 cells were pre-

incubated with alkaline phosphatase, which desphosphorylates phosphorylated proteins for 30 min (ALP, 20 U/ml). Pre-incubation with ALP in fact prevented the appearance of high molecular weight NR4A2 bands upon P/I treatment, suggesting that phosphorylation is involved in NR4A2 PTMs (Fig 3C). However, incubation of the cell lysate with ALP did not modify the pattern of NR4A2 modification, indicating that phosphorylation is required for ubiquitination, but not for the retention of the ubiquitin molecules on NR4A2.

Since ubiquitin acts as a tag for proteosomal degradation, we next wanted to assess if proteasome blockage with MG132 affect the abundance of ubiquitinated NR4A2 species. Indeed, pre-incubation with 1 μM of MG132 for 2 h, resulted in a remarkable accumulation of high-molecular weight NR4A2 6 h after treatment with P/I. Furthermore, 24 h after treatment ubiquitinated NR4A2 signals were also stronger, suggesting that proteasome inhibition prolongs NR4A2 turnover (Fig 3D). Altogether these results demonstrate that P/I induces both upregulation and phosphorylation dependent ubiquitination of NR4A2 and that proteasome inhibition seem to stabilize the modified NR4A2.

Post-translational modifications induce NR4A2 accumulation in the endoplasmic reticulum and endolysosomes

Beyond their role as transcription factors, nuclear orphan receptors 4A have been associated with alternative functions in different cellular organelles. Therefore, we wanted to examine whether ubiquitination of NR4A2 could influence its localization within the cell. Whole cell lysates were separated into nuclear and cytoplasmic fractions, and western blots were performed with α-NR4A2 antibody (Fig 4A). Nucleo-cytoplasmatic fractionation efficiency was confirmed both with α-tubulin (cytoplasmic) and α-PARP-1 (nuclear). This analysis showed that upon P/I treatment, the ubiquitinated NR4A2 (77KDa) predominantly localized to the cytoplasm. Interestingly, a much weaker band bellow the 77KDa band was detected in the nuclear fraction both in untreated and P/I-treated cells, suggesting that an alternative PTM pattern might be required for nuclear localization. However, unlike the 77KDa band, this nuclear NR4A2 fraction was not influenced by P/I treatment.

To further characterize the cytoplasmic localization of NR4A2, mitochondrial (MIT), endoplasmic reticulum (ER) and lysosome (LYS) fractions were analysed 6 h after P/I treatment. MnSOD, PERK and LAMP1 antibodies were used as subcellular markers

of each of these fractions, respectively (Fig 4B). Ubiquitinated NR4A2 was predominantly detected in the ER and lysosomal fractions, while no NR4A2 signals were detected in the mitochondrial fraction. In addition, the localization patterns of NR4A2 induced by P/I with or without MG132 pre-treatment both at 6 and 24 h were analysed (Fig 4C). While MG132 pre-treatment strongly enhanced the early (6 h) accumulation of high-MW NR4A2 both in ER and lysosome, weak NR4A2 signals could be detected exclusively in the lysosomal fraction at later time points (24 h).

In order to further characterize NR4A2 localization in the LAD-2 cells, NR4A2 trafficking was analysed by immunofluorescence with confocal microscopy. While no NR4A2 signals could be detected either in nucleus or cytopplasm, after 6 h P/I treatment NR4A2 positive foci could be detected in the cytoplasm, as well as a much weaker and difuse nuclear signal (Fig 5A). The identiy of these signals was confirmed by NR4A2 silencing, and MG132 pretreatment increased NR4A2 signals by 31% at this time point (Fig 5B). To confirm co-localization of NR4A2 to ER, LAD-2 cells were incubated with α-NR4A2 and α-calnexin (ER marker). 41% of P/I-induced NR4A2 colocalized with calnexin, while MG132 pre-treatment increased co-localization to 62% (percentage of total NR4A2 signal). Next, LAD-2 cells were transfected with eGFP-Rab5 (early endosome marker), eGFP-Rab7 (late endosome marker) or eGFP-LAMP1 (lysosomal marker), co-stained with α-NR4A2. While the expression of eGFP-Rab5, eGFP-Rab7 and eGFP-LAMP1 remained unchangedwith all treatments tested, quantitative colocalization analysis revealed that 6 h after P/I treatment 18% of the NR4A2 cytoplasmic signals colocalized with Rab5, 21% with Rab7 and 17% with LAMP1 (Fig 5C). Pretreatment with MG132 further enhanced NR4A2 colocalization to the endolysosomal compartment to 37%, 32% and 26% to the early endosome, late endosome and lysosome, respectively.

The previously decribed cell fractionation studies suggested that MG132 affects the turnover rate of NR4A2, resulting in increased accumulation of this factor in lysosome 24 h after P/I tretament (in the lysosome, Fig 4C). Therefore, we wanted to confirm the distribution pattern of NR4A2 in the different compartments by immunofluorescence at this late time point (Fig 5D). In agreement with western blot observations, NR4A2 IF signals were much weaker at this time point, which could not be mapped to the early endosome, late endosome or lysosome. However, MG132 pretreatment enchanced NR4A2 signals by 17% which colocalized with Rab 7 (12%), cand LAMP1 (7%).

Taken together, these results indicate that P/I-induced ubiquitination consititues a signal for NR4A2 localization to the endoplasmic reticulum, early and late endosomes, and lysosomes, and that proteasome inhibition stabilizes ubiquitinated NR4A2, resulting in increased levels of this protein in these cytoplasmic structures 6 h after P/I treatment and, to a lesser extent, at a late time points (24 h).

NR4A2 mediates PMA and Ionomycin-induced IL-4

We next wanted to establish the biological relevance of NR4A2 ubiquitination. Therefore, we first wanted to identify cytokines whose expression depends on NR4A2. Real time RT-PCR was carried out using a human chemokine RT-PCR array that allowed assessing the transcription of 90 human chemokines and chemokine receptor genes. This screening revealed 18 gene products that were up-regulated in LAD-2 cells by P/I, 6 h after treatment (Suppl Fig 3). Remarkably, the induction of 8 of these gene products could be significantly reversed by silencing NR4A2, suggesting that the expression of these chemokines depend on this factor. The effect of NR4A2 silencing was very pronounced for interleukin-4 (IL-4), a cytokine involved in T-cell differentiation into Th2 effector cells in allergen-driven responses. Therefore, this interleukin was selected for further analysis. RT-PCR confirmed the upregulation of this cytokine upon 6 h P/I stimulation (936-fold, as compared to untreated cells), which could be largely reversed by NR4A2 knockdown (81.6-fold, Fig 6A). Next, we wanted to confirm whether NR4A2 silencing also affects the release of IL-4 to the cellular milieu. Treatment with P/I indeed induced robust IL-4 release to the cell culture medium, from 39 pg/ml (unstimulated) to 423 pg/ml 6 h after treatment and to 1152 pg/ml 24 h after treatment (11-fold and 29-fold respectively). Interestingly, the concentration of IL-4 was significantly decreased by NR4A2 silencing (Fig 6B), although this effect most more pronounced at the early time point (6 h: 63% reduction; 24 h: 25% reduction). Next, we wanted to identify the signaling kinases involved in IL-4 induction. In a previous report we showed that AR-mediated NR4A2 upregulation involved PKC, MEK and ERK kinases [1-3] . Here showed that blockage of either PKC or MEK with GF109203X (10 μM) or PD98059 (100 μM) respectively partially reverted P/I-induced IL-4 release 6 h (Fig 6C), but not 24 h after P/I stimulation. Next we evaluated P/I stimulation activated ERK phosphorylation. Indeed, ERK1/2 was phosphorylated 6 h after P/I treatment, and it return to basal

levels by 24 h. Interestingly, although MG132 pre-treatment further enhanced ERK1/2 phosphorylation at the early time point (Fig 6D), NR4A2 silencing did not affect ERK1/2 phosphorylation. Altogether, these results indicate that early release of P/I-induced IL-4 (6 h) is mediated by PKC and ERK kinases and largely dependent on NR4A2, although the late release of IL-4 (24 h) appears to involve additional signaling pathways.

NR4A2 stabilization stimulates early IL-4 release but inversely correlates with late IL-4 concentrations

Based on the observation that NR4A2 silencing extensively prevented IL-4 induction, particularly at an early time point, and that pre-treatment with MG132 enhanced NR4A2 abundance, we reasoned that proteasome inhibition might result in increased levels of these cytokine. To assess this hypothesis the effect of MG132 pre-treatment on IL-4 induction and release was assessed 6 h and 24 h after P/I treatment. At the transcriptional level, MG132 pre-treatment further upregulated IL-4 6 h after P/I treatment by 37.5% (1255-fold), although this effect was not longer observed 24 h after treatment (Fig 7A). Similarly, MG132 pre-treatment induced a robust release of IL-4 6 h after treatment, which reached 789.4 pg/ml (102%) higher than without MG132 pre-treatment), suggesting a casual relationship between the accumulation of NR4A2 and IL-4 release (Fig 7B). However, MG132 reduced the late release of P/I-induced IL-4 induction by 59.0 % (from 1416.6 pg/ml to 581.5 pg/ml), suggesting a biphasic effect of proteasome inhibition on IL-4 synthesis. The fact that a reduction of IL-4 release was observed, despite the stabilization of NR4A2 by MG132, suggests that late P/I-induced late release of IL-4 may depend on another factor.

NR4A2 and NF-κB control the early and late IL-4 release, respectively

Since proteasomal degradation of IκB is required for the activation of NF-κB [30], we reasoned that this signaling pathway could be involved in late phase IL-4 release. To address this question we first wanted to assess the transcritional activity of NR4A2 by means of the NBRE-LUC reporter assay both 6 and 24 h after P/I treatment LAD2 cells were transiently co-transfected with tk-NBREx3-luc and the pRL-TK plasmids (transfection normalization), stimulated with P/I and luciferase/renilla ratios calculated (Fig 8A). This analysis revealed a strong induction of the NBRE reporter at

both time points (7.0 and 8.7-fold against untreated, respectively). As expected, MG132 pre-treatment resulted in further increase in these signals (12.5 and 15.7-fold). In order to establish whether nuclear factor NF-kappa-B (NF-κB) activation conditions NR4A2 response, NBRE transcriptional activation was assessed after silencing of NF-κB p65 subunit. This assay revealed a strong NBRE activity independent of p65 at an early time point. However, 24 h after exposure p65 silencing resulted in a significant decrease in NBRE activity (31.5%), indicating a late cross-talk between both transcription factors.

Conversely, we next wanted to establish whether NR4A2 is required for NF-κB activation. Therefore, LAD-2 cells were co-transfected with the wt-κB-LUC and the Renilla plasmids and transcriptional acitivity calculated as described (Fig 8B). P/I induced a robust luciferase responseboth 6 h and 24 h after P/I treatment (20.4-fold and 44.5-fold, respectively). As expected, p65 silencing completely blocked the induction of this reporter system. Also, this reponse strongly blocked by the NF-κB inhibtor BAY11-7082 (56.7% reduction at 6 h and 56.3% reduction at 24 h), and by MG132 pre-treatment, indicating that an intact proteasome is indeed required for NF-κB activation. However, NR4A2 silencing revealed a dual effect on NF-κB activity; while early NF-κB activity was independent of this orphan receptor, wt-κB -LUC 24 h after P/I stimulation was almost completely prevented by NR4A2 silencing, indicating that late NF-κB is dependent on NR4A2.

To further characterize the downstream effects of NR4A2 and NF-κB signaling, we next assessed early and late IL-4 release (Fig 8C). In contrast to NR4A2 silencing, knockdown of p65 did not affect the release of IL-4 6 h after P/I treatment, confirming that the early IL-4 response depends on NR4A2. However, 24 h after P/I stimulation, silencing p65 had a dramatic effect in IL-4 release (from 1373.8 to 668.3 pg/ml). In addition, treatment with BAY11-7082 also resulted in a remarkable reduction of IL-4 (from 1373.8 to 467.5 pg/ml), which was almost identical to the inhibition by MG132, indicating that NF-κB is required for the late phase response. Interestingly, the 24 h decrease in NF-κB activity observed by NR4A2 silencing (Fig 8B) correlated with a partial reduction of IL-4 at this late time point, suggesting that late NF-κB responses require a functional NR4A2. Altogether, these results confirm that while early IL-4 response is highly dependent on NR4A2, the involvement of NF-κB signaling in the late IL-4 release (24 h), in an NR4A2-dependent way.

Adenosine receptor A_{2A} modulates NR4A2 expression, post-translational modifications and IL-4 release

Activation of the adenosine receptor A_{2A} (A_{2A}AR) has been linked to inflammation suppresion and tissue remodelling [23, 31] and recently we observed that the stimulation of this A_{2A}AR modulates NR4A2 induction by the adenosine analogue NECA and by PMA in human mast cells. Therefore, we now wanted to assess whether A_{2A}AR activation could play a similar modulatory role on P/I-induced NR4A2 ubiquitination and downstream signaling. RT-PCR analysis revealed that pre-treatment with 1 μM of the A_{2A}AR agonist CGS-21680 reduced P/I-mediated NR4A2 upregulation from 1120.7 fold to 575.7 fold (48.6% reduction), 6 h after treatment (Fig 10A). Furthermore, western blot analysis confirmed that CGS-21680 negatively influenced NR4A2 ubiquitination (Fig 10B) and also that A_{2A}AR silencing exacerbated NR4A2 high molecular weight signlas, indicating that this AR receptor subtype negatively regulates NR4A2 ubiquitination. Next, we wanted to assess whether A_{2A}AR-mediated decrease of NR4A2 post-translational modifications correlates with a decrease in NR4A2 localization to cytoplasmic organelles. Indeed, immunofluorescence confirmed that CGS21680 significantly reduced the number and intensity of NR4A2 foci (42.1%) (Fig 10C).

Finally, we wanted to establish whether CGS-21680 modulatory effect on NR4A2 signaling was associated with decreased NBRE and NF-κB activity and IL-4 release. Luciferase reporter assays indeed confirmed that CGS-21680 pre-treatment caused a significant reduction both in P/I-induced NBRE-LUC activity at 6 h (49.8%) and on P/I-induced NF-κB activity at 24 h (wt-κB -LUC, 38.1%) (Fig 10D), and these changes correlated with a significant decrease of IL-4 release 6 and 24 h after P/I treatment (48.7 and 53.0%, respectively; Fig 10E). In summary, these experiments show that activation of the A_{2A}AR can modulate IL-4 release through downregulation of NR4A2 signaling and the associated NF-κB activity.

Discussion

In recent years the role of mast cells as regulators of immune responses have become better understood. Beside their involvement in immediate release of preformed inflammatory mediators in allergic responses, it is now widely accepted that mast cells can sustain inflammation through the can differential release of inflammatory

mediators [32]. Our previous work identified a remarkable upregulation of NR4A2 and NR4A3 receptors in HMC-1 mast cells in response to adenosinergic signaling [1], suggesting that this family of receptors could represent broader markers of mast cell activation. In fact, NR4A1 upregulation and phosphorylation has been reported in response to different mast cell stimuli [33]. The goal of this study was to further characterize NR4A2 pro-inflammatory signaling and downstream effectors in mast cells, and to assesses the effect of the antiinflammatory adenosine receptor A_{2A}AR on this pathway.

Nuclear orphan receptors 4A are structurally related to nuclear hormone receptors and have been shown to function as ligand-independent transcription factors expressed in a wide variety of metabolically active tissues [2, 34]. Because they are constitutively active transcription factors, activity of NR4As is tightly controlled at the level of expression, subcellular localization and post-translational modification [35]. NR4A2 can be phosphorylated by ERK2 at S126 and T132, and these post-translational modifications appear to play a role in regulation in the expression of the tyrosine hydoxylase [36]. More recently, phosphorylation of NR4A2 by DNA-PK has been shown to be required for proficient DNA double-strand break repair [13]. NR4A2 has 2 putative sumoylation sites at lysines 91 and 577 [14], although no sumoylation, ubiquitination or methylation of NR4A2 have yet been identified.

The first main finding of the study was the identification of phosphorylation-dependent ubiquitination of NR4A2 in mast cells, upon the concomitant treatment with PMA and ionomycin. First, we observed by western blot analysis that P/I stimulation led to a high molecular band of approximately 75KDa, while the expected size of full length NR4A2 is 66KDa. Second, the appearance of this high molecular band could be inhibited UBEI-41, an inhibitor of the ubiquitin E1 enzymes, which catalyse the first step in the ubiquitination reaction. Third, immunoprecipitated NR4A2 specifically cross-reacted with ubiquitin antibodies, resulting in a signal suggestive of (poly)ubiquitination of about 75KDa. Fourth, proteasome inhibition with MG132 enhanced this high-molecular weight band both 6 h and 24 h after P/I treatment, suggesting that ubiquitination directs NR4A2 to proteasomal degradation. In addition, while treatment of cell lysates with alkaline phosphatase did not affect the ubiquitination pattern, pre-incubation with this phophatase significantly reduced ubiquitination of NR4A2, indicating that phosphorylation preceeds ubiquitination.

Previous studies showed that phosphorylation of NR4A1 regulates nuclear export, and

cytoplasmic and mitochondrial localization [37]. Here we report that ubiquitinated NR4A2 locates in cytoplasma. Furthermore, cell fractionation and confocal microscopy allowed us to determine that unlike phosphorylated NR4A1, ubiquitinated NR4A2 localizes to ER, endosomes and lysosomes. Furthermore, proteasome inhibition with MG132 enhanced NR4A2 signals in these organelles 6 h after P/I stimulation, and prolonged the residence time of NR4A2 in the endolysosomal compartment (signals could be detected 24 h after P/I treatment at the later) . Secretory lysosomes in granulocytes are most often preformed structures storing secretory products while awaiting stimulation [13, 38]. Our result strongly suggested NR4A2 translocation to lysosome as a stimulation signal of the preformed IL-4 at the early phase.

The second main finding in this study was the identification of a sustained, NR4A2-dependent IL-4 release upon P/I treatment in mast cells. By a combination of silencing and reporter assay approaches, we showed that the early IL-4 response (6 h after P/I treatment depends on NR4A2. Proteasome inhibition could further increase both the tk-NBREx3-luc reporter activity and early IL-4 release. On the contrary, late IL-4 release did not correlate with NBRE activity, but with NF-κB activity as assessed with the wt-κB reporter assay. Three independent approaches support this hypothesis. First, in agreement with previous studies that demonstrated that MG132 prevents IκB degradation, a step required for NF-κB translocation into the nucleus [39], pre-treatment with MG132 resulted in a reduced late IL-4 release. Second, NF-κB inhibition with BAY11-7082 resulted in a similar effect. Third, silencing of NF-κB subunit p65 almost completely abolished the release of this cytokine (Fig 8C).

Interestingly, although NF-κB is already activated at an early time point, this transcription factor is not required for this early response, since at this time point neither p65 silencing nor MG132 pretreatment affected P/I-induced IL-4 levels (Fig 8C). On the contrary, while NR4A2 silencing did not affect early NF-κB it had a very marked effect both on NF-κB activity and IL-4 release 24 h after P/I tratment (Fig 8B and 8C). These results suggest 2 phases of NF-κB activativity: an early phase, independent of NR4A2 and not required for early IL-4 release, plus a later phase dependent on NR4A2 and capable of driving IL-4 release at this stage. Several studies have investigated the cross-talk between NR4As and NF-κB. Both NR4A1 and NR4A2 have been proposed as a downstream target of NF-κB in response to

inflammatory mediators [40, 41]. More recently, NR4A2 has been shown to contribute with NF-κB towards the expression of IL-8 [42]. The data presented here complements these forms of cross-talk between NR4A2 and NF-κB, suggesting that P/I treatment generates a signal from NR42 to NF-κB, at least in the late time points (24 h). However, this data does not allow to conclude whether this effect is mediated by the direct upregulation of NF-κB subunits, or whether NF-κB activation is indirectly linked to NR4A2 signals such as NR4A2-induced ER stress. Here we show that the accumulation of ubiquitinated NR4A2 in the ER leads to the induction of CHOP, a surrogate of ER-stress [43, 44]. Interestingly, ER stress can lead to NF-κB activation by at least 3 different pathways: through TRAF2 dependent IKK phosphorylation; through PERK-induced phosphorylation of eIF2α**,** which decreases IκB protein by repression of IκB translation, leading to interference with the export of nuclear NF-κB to the cytoplasm; or through ATF6, which causes activation of NF-κB via the Akt pathway [45]. Further studies will be required to establish the relevance of NR4A2-mediated ER stress in late NF-κB activation and IL-4 induction.

IL-4 is a prototypic immunoregulatory cytokine responsible for initiating allergic responses and it essential is uniquely required to polarize naïve helper cells (Th0 cells) to a type 2 phenotype, characterized by secretion of IL-4 itself, plus IL-5, IL-9, IL-10, and IL-13. IL-4 also induces B cells to switch their immunoglobulin production to IgE secretion. Mast cells and basophils readily produce IL-4 after crosslinking of their FcεRI receptors [46]. Here we present a novel link between P/I activation of mast cells and IL-4 release, and we show that the late release of IL-4 depends on NR4A2-induced NF-κB activity, suggesting that although proteasome inhibition enhances early IL-4 release, it could potentially translate in downregulation of sustained, IL-4 mediated allergic responses in vivo.

Finally, we showed the inhibitory effect of the adenosine receptor A_{2A} on NR4A2 and NF-κB dependent IL-4 induction. Although several lines of evidence support the anti-inflammatory effect of the A_{2A}AR, the mechanism behind these effects have remained elusive. *In vitro* studies using endothelial cells have indicated that the ability of the A_{2A}AR to exert such wide-ranging anti-inflammatory effects could be explained by its ability to regulate pro-inflammatory signaling pathways [47, 48]. In addition, it has previously been shown that that activation of T lymphocytes can be suppresed by A_{2A}AR activation [49], and that CGS21680 could succesfully suppress asthmatic

inflammation in animal models [31]. Therefore, the data presented here suggests that through the modulation of IL-4 release, A_{2A}AR activation in mast cells can negatively influence the development of allergic Th2 responses.

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Figure 1A

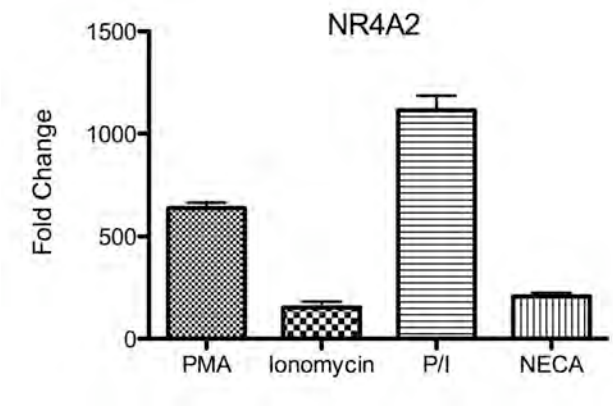


Figure 1B

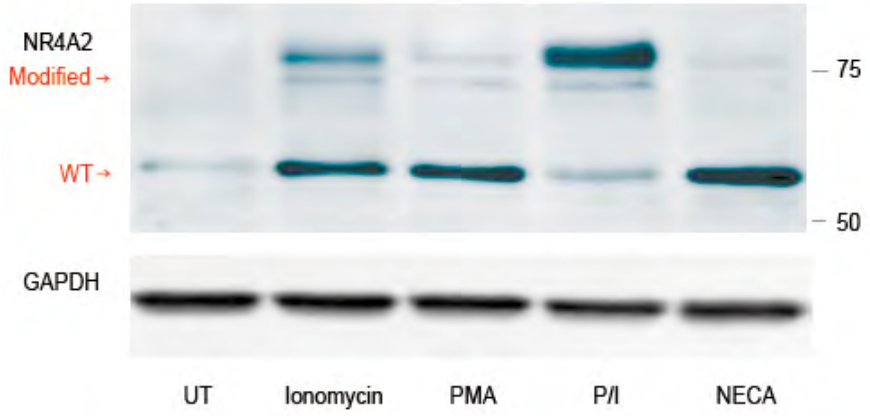


Figure 1. P/I-induced NR4A2 expression (A) RT-PCR shows NR4A2 induction at 2 h by PMA (25 nM), Ionomycin (1 µM), combination of PMA with ionomycin (P/I) and NECA (10 µM). Fold changes are calculated in relation to untreated controls. Values represent the means of three experiments ± SEM (B) Western blot analysis of NR4A2 expression. Cell extracts of untreated, treated with 0.1 µM ionomycin, 25 nM PMA, P/I and 10 µM NECA for 6 h, respectively. GAPDH is included as an internal control

Figure 2

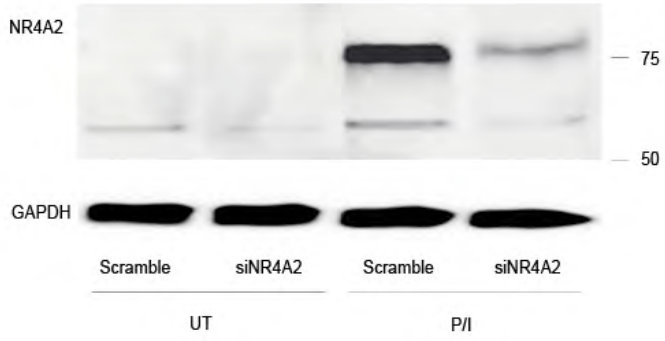


Figure 2. Transfection of LAD-2 cells with NR4A2 siRNA for 48 h. Western blot shows specific downregulation of NR4A2 expression induced by P/I. Scramble siRNA was included as a control. GAPDH is included as an internal control

Figure 3A

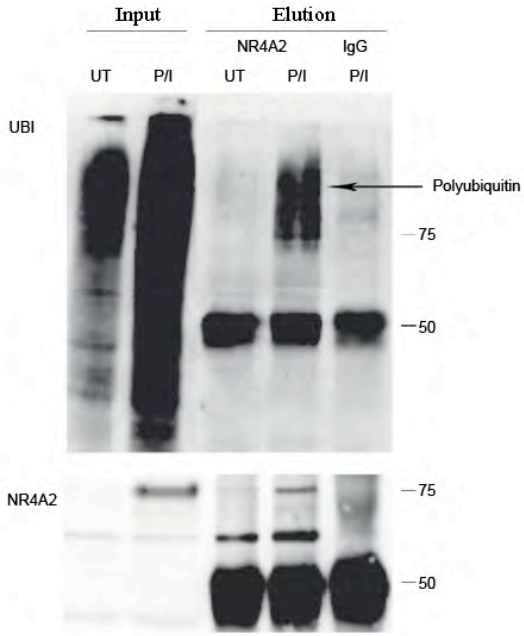


Figure 3B

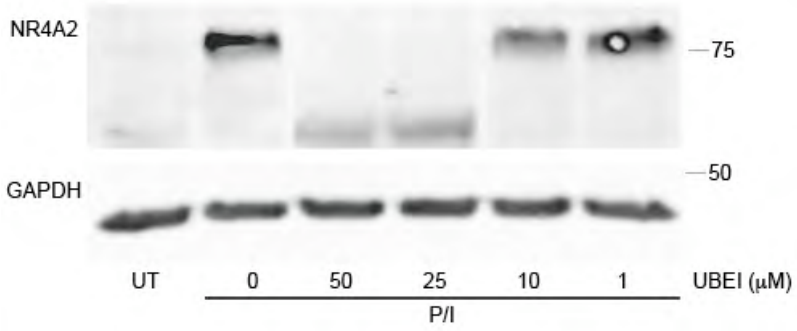


Figure 3C

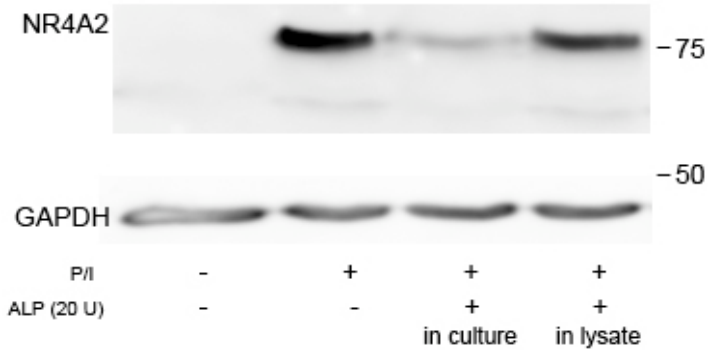


Figure 3D

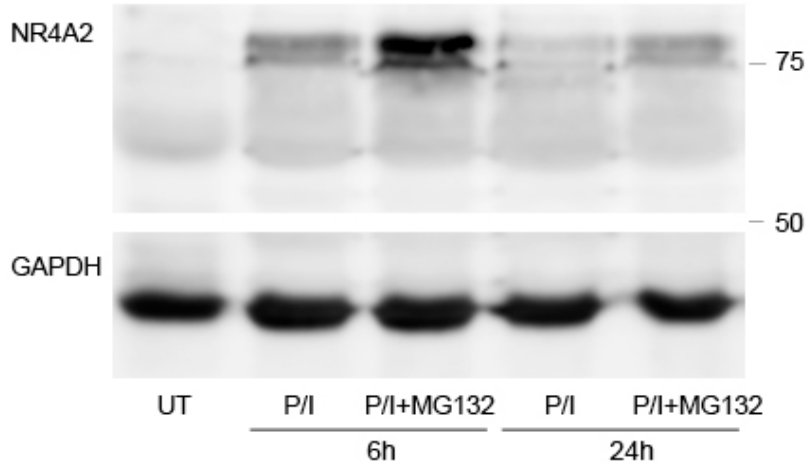


Figure 3. Phosphorylation-dependent ubiquitination of NR4A2 (A) LAD-2 cells were treated with or without P/I for 6 h. The total protein lysate was immunoprecipitated using anti-NR4A2 antibody or IgG control. Probing blots with anti-ubiquitin antibody revealed ubiquitinated NR4A2 by P/I. Migration of molecular mass standards is indicated in KDa (B) Pretreatment of ubiquitin E1 ligase inhibitor (UBEI) for 2 h, western blot shows the reversion NR4A2 induction by P/I in a dose-dependent manner (C) Phosphorylation dependent ubiquitination of NR4A2. LAD-2 was pretreated with alkaline phosphatase (ALP, 20 U) in cell culture or applied in protein lysate with P/I. (D) LAD-2 was pretreated with MG132 (1 μM) for 2 h. Total protein was harvested after 6 h or 24 h with P/I treatment. GAPDH is included as an internal control

Figure 4A

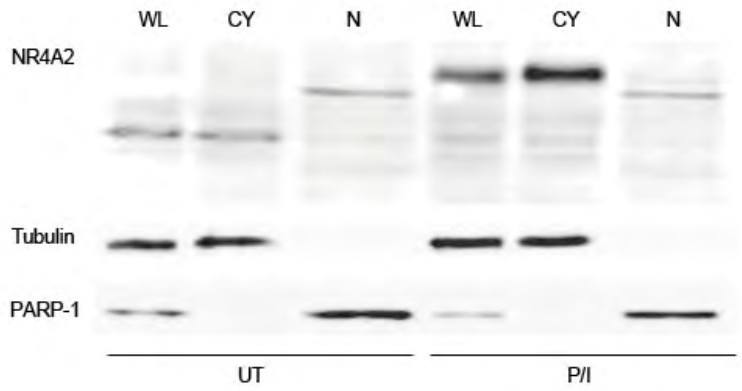


Figure 4B

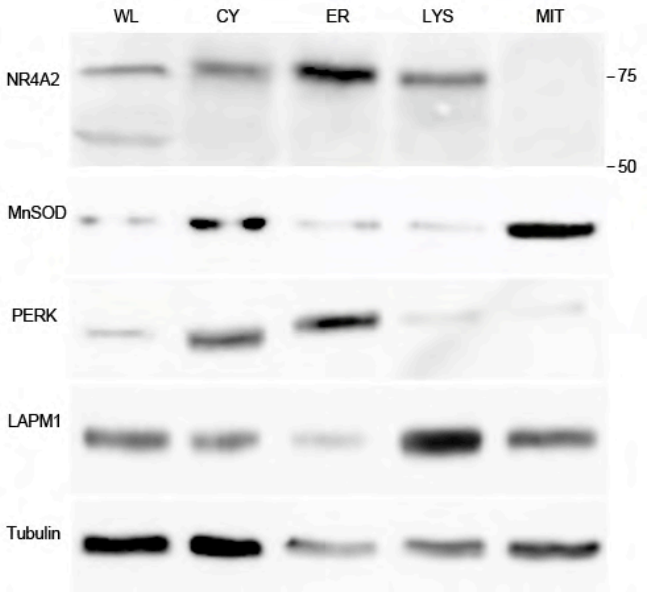


Figure 4C

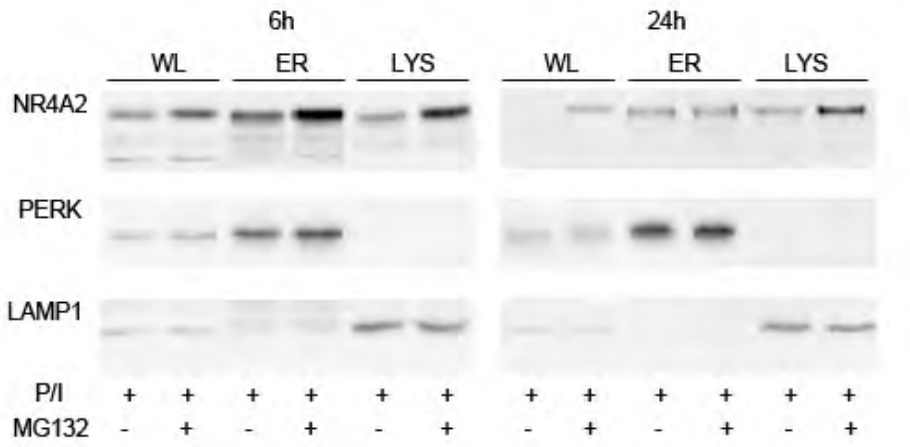


Figure 4. NR4A2 was located at endoplasmic reticulum (ER) and lysosome. Subcellular fractionation of NR4A2 obtained by differential centrifugation and analyzed by western blot. LAD-2 cells were treated with P/I and protein was harvest after 6 h (A) Fractions obtained during the purification of cytoplasmic and nuclear were analyzed by western blot. Tubulin and PARP-1 were using as the markers for cytoplasmic and nuclear (B) Mitochondrial, endoplasmic reticulum (ER) and lysosome fractions were isolated. MnSOD, PERK and LAMP1 were using for cell compartmental markers, respectively (C) Fractionation by sucrose density or Percoll centrifugation. LAD-2 cells were pretreated with MG132 and treated with P/I for 6 or 24 h. Equal volumes of each fraction were evaluated by western blot analysis for the presence of PERK and LAMP-1

Figure 5A

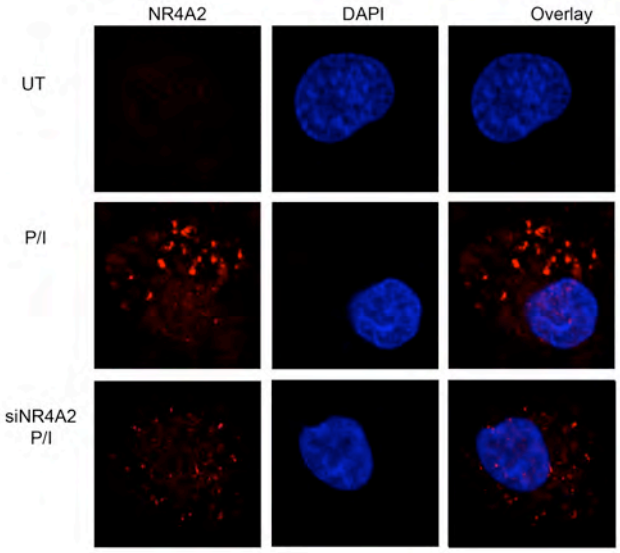


Figure 5B

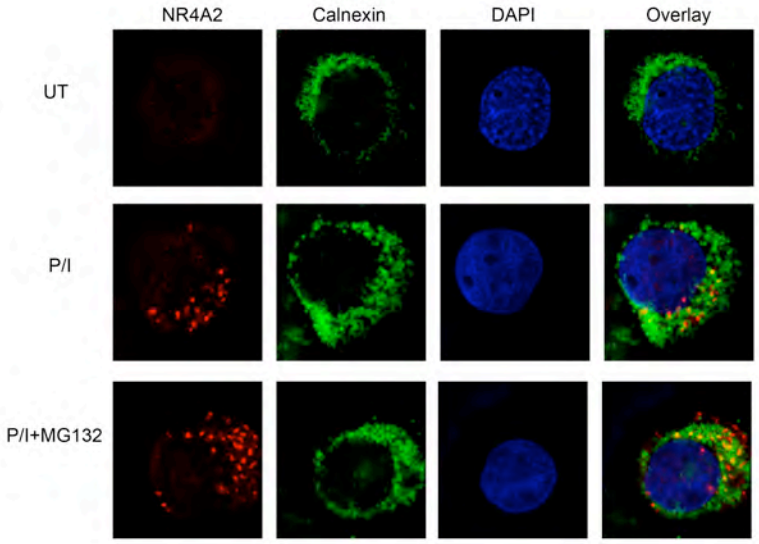


Figure 5C **Figure 5D**

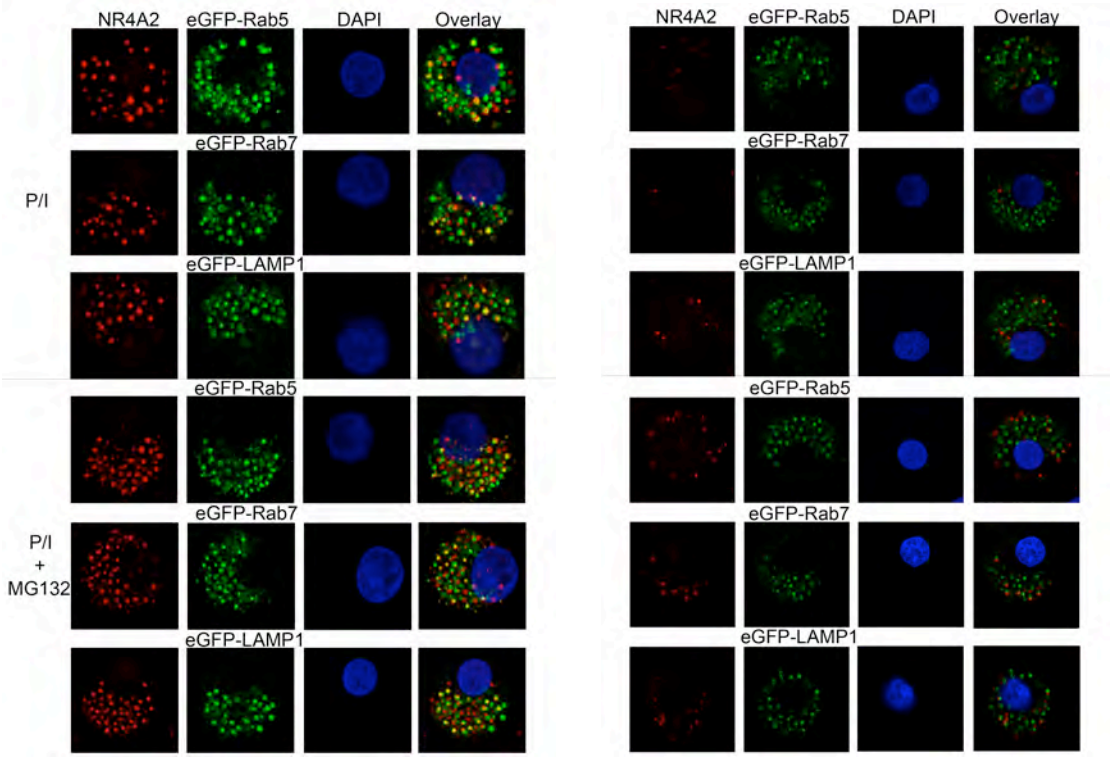


Figure 5. Immunofluorescence analysis of NR4A2 in ER and endo-lysosomal system (A) LAD-2 cells were transfected with specific siNRA of NR4A2 gene for 48 h, and then stimulated with P/I for 6 h. Immunofluorescence showed the foci of NR4A2 (B) LAD-2 cells were treated as indicated in the figure. Immunofluorescence showed the colocalization of NR4A2 with calnexin (ER marker) (C, D) LAD-2 cells were transfected with eGFP-Rab5 (early endosome marker), eGFP-Rab7 (late endosome marker) or eGFP-LAMP1 (lysosome marker) for 24 h. Treated LAD-2 cells as indicated in the figure for 6 h (C) or 24 h (D). Immunofluorescence showed the colocalization of NR4A2 with endolysosomes

Figure 6A

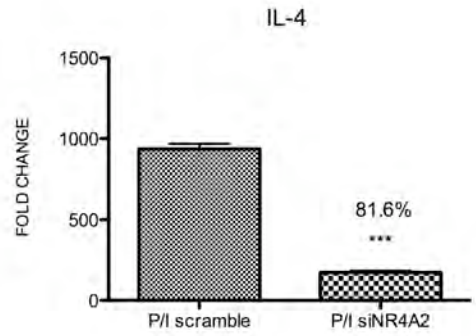


Figure 6B

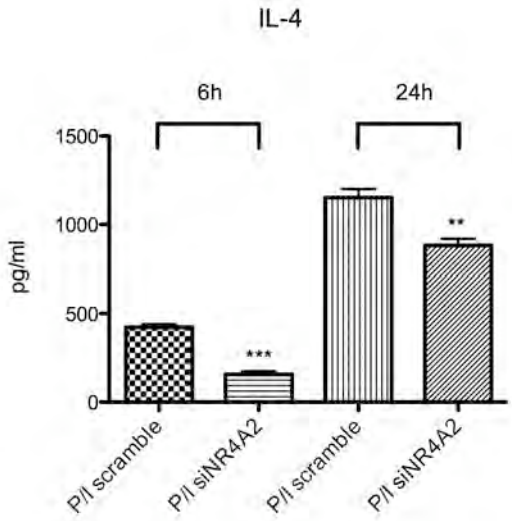


Figure 6C

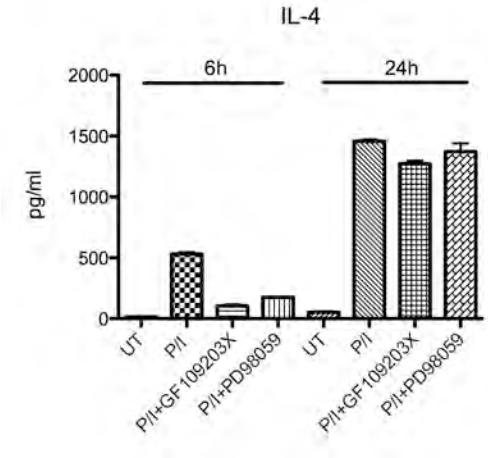


Figure 6D

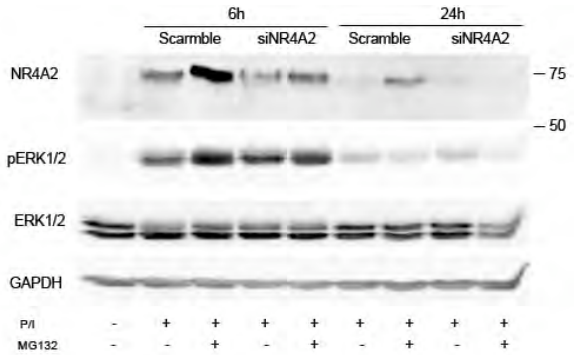


Figure 6. NR4A2 mediates PMA and Ionomycin-induced IL-4 at early phase. LAD-2 were transfected with scramble RNA or NR4A2 siRNA for 48 h. (A) Real-time PCR analysis on the induction of IL-4 in LAD-2 cells stimulated with P/I for 6 h. The data were normalized to the housekeeping gene (β-actin gene). Fold changes are calculated against untreated controls (B) The culture supernatants were collected after 6 or 24 h. IL-4 concentrations determined by ELISA. Values represent the means of three independent experiments ±SEM (C) LAD-2 cells were pretreated GF109203X (10 μM) or PD98059 (100 μM) for 30min, following treatment for 6 h as indicated in the figure. The culture supernatants were collected IL-4 concentrations determined by ELISA. Values represent the means of three independent experiments ±SEM (D) Transfection of LAD-2 cells with NR4A2 siRNA for 48 h, treated with P/I with or without MG132 for 6 or 24 h as indicated. NR4A2, pERK1/2 and total ERK1/2 expression were shown. ERK1/2 and GAPDH is included as an internal control

Figure 7A **Figure 7B**

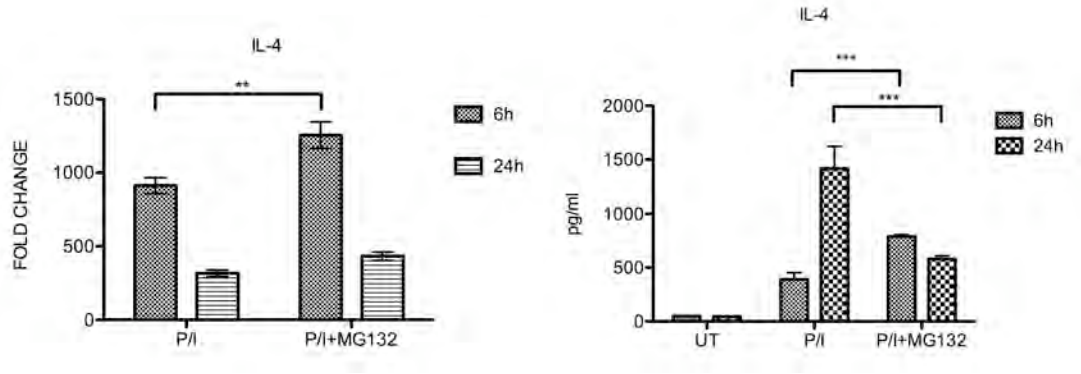


Figure 7. Effect of NR4A2 PTM on IL-4 expression. LAD-2 were cultured in the presence or absence of the proteasome inhibitor MG132 (1 μM) for 2 h and stimulated with P/I for 6 or 24 h (A) Real-time PCR analysis on the induction of IL-4. The data were normalized to the housekeeping gene (β-actin gene). Fold changes are calculated against untreated controls (B) The culture supernatants were collected IL-4 concentrations determined by ELISA. Values represent the means of three independent experiments ±SEM

Figure 8A

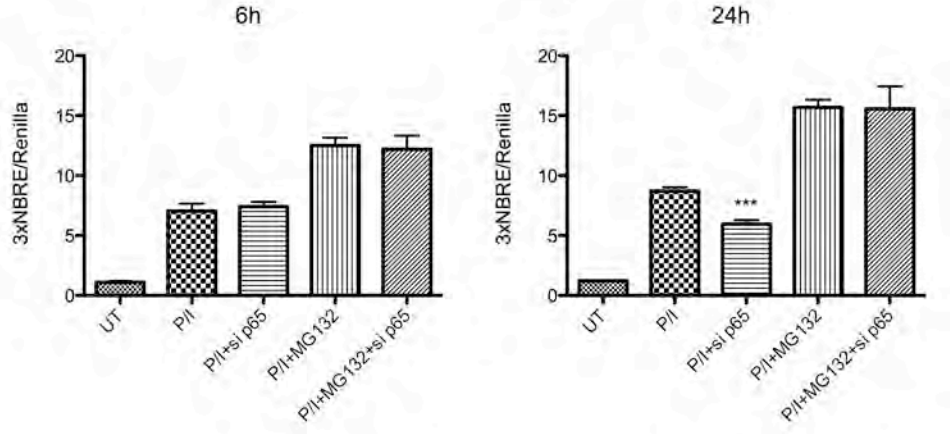


Figure 8B

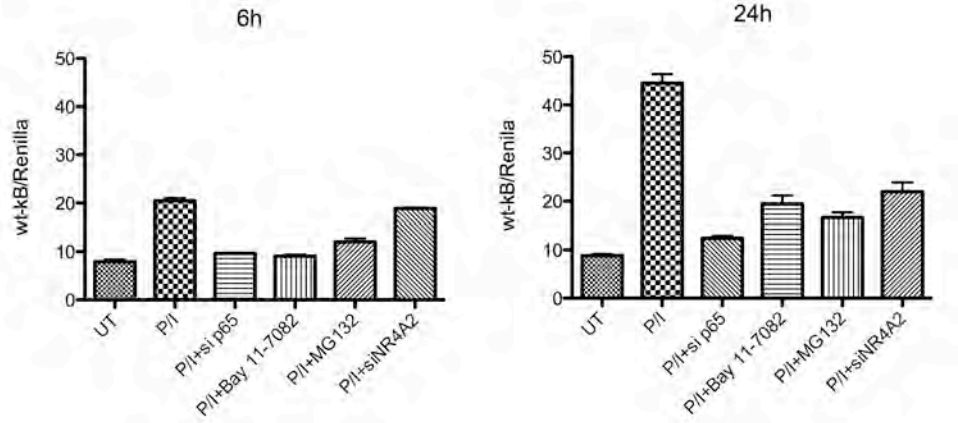


Figure 8C

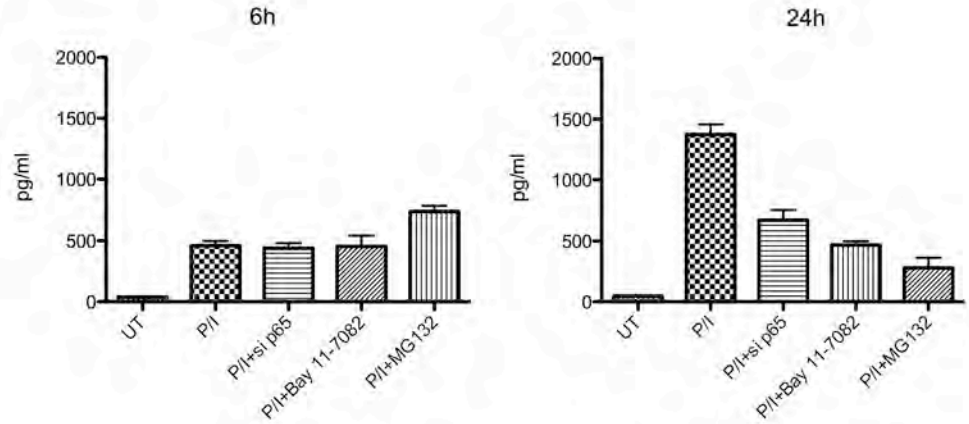


Figure 8. Effect of NF-κB signaling on IL-4 expression at late phase (A) Co-transfected of LAD-2 with 3xNBRE and pRL-TK vector (renilla plasmids) for 24 h. Treated with P/I with or without MG132 for 6 or 24 h as indicated. Firefly 3xNBRE/renilla ratio was shown (B) Co-transfected of LAD-2 with wt-κB and pRL-TK vector for 24 h. The cells were pretreated Bay 11-7082 (10 μM) for 1 h, following treatment for 6 or 24 h as indicated in the figure. Means and ±SEM of the firefly/renilla ratios of at least three independent experiments are shown (C) Transfection of LAD-2 cells with p65 siRNA for 48 h. Pretreated with Bay 11-7082 (10 μM) for 1h, following treatment for 6 or 24 h as indicated in the figure. The culture supernatants were collected IL-4 concentrations determined by ELISA. Values represent the means of three independent experiments ±SEM

Figure 9A

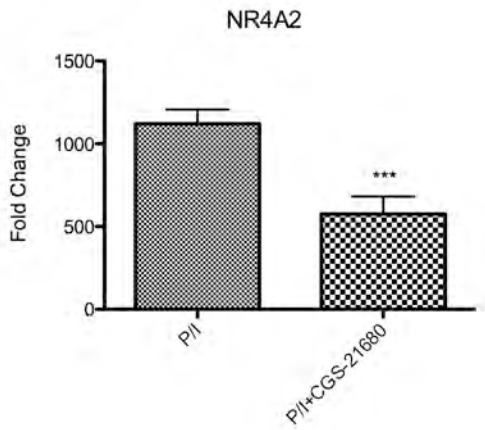


Figure 9B

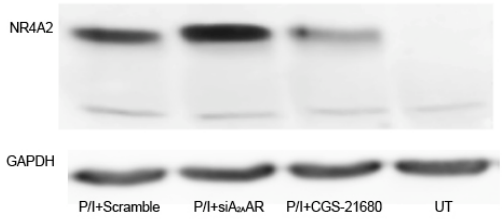


Figure 9C

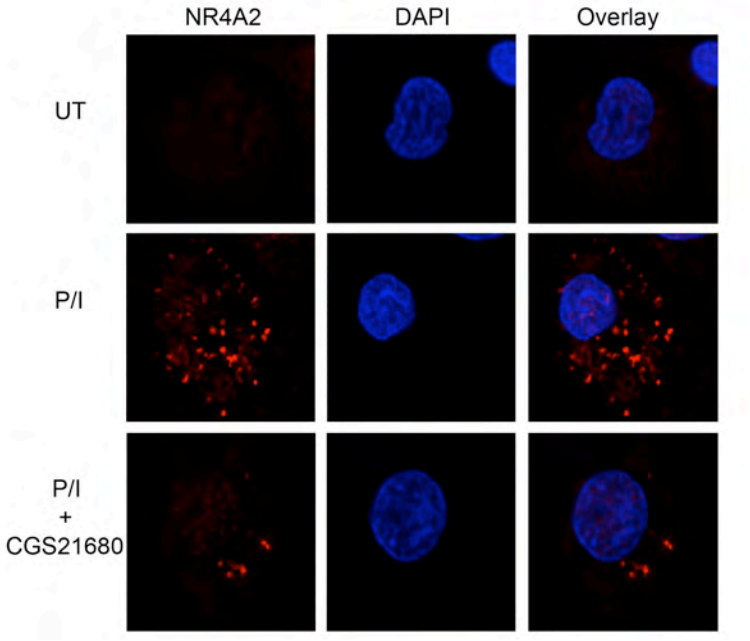


Figure 9D

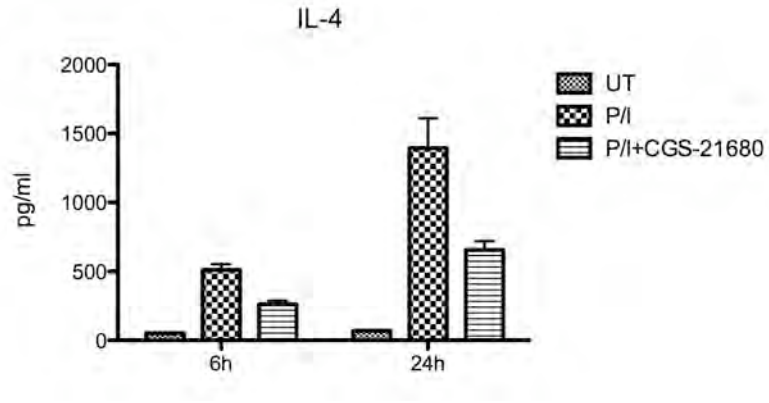


Figure 9E

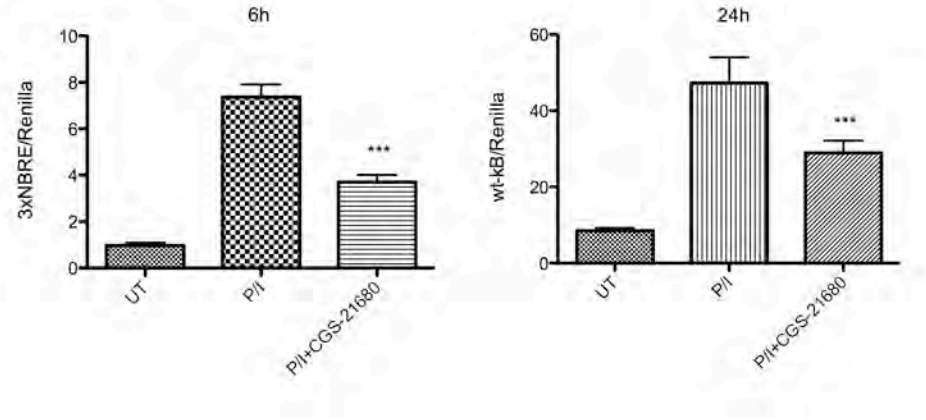
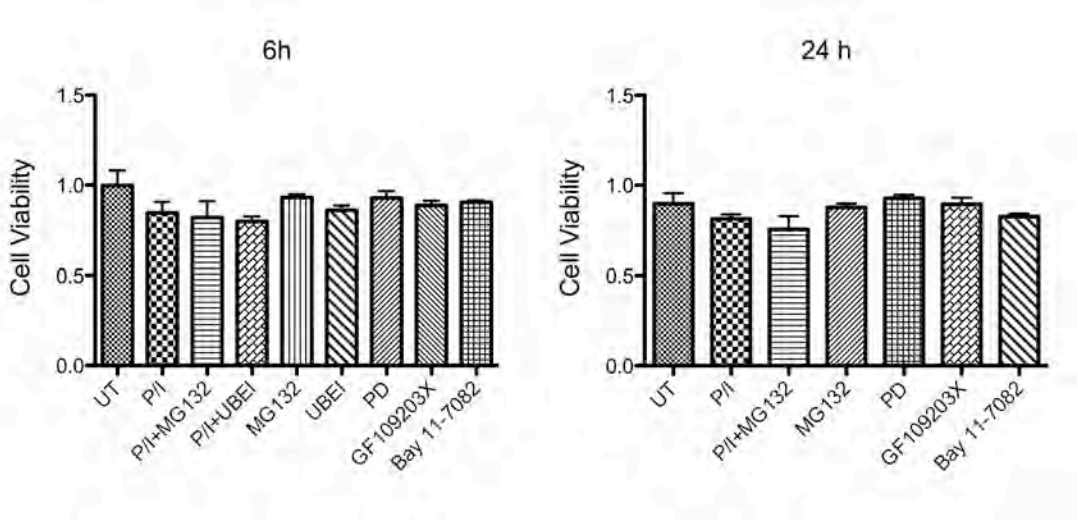


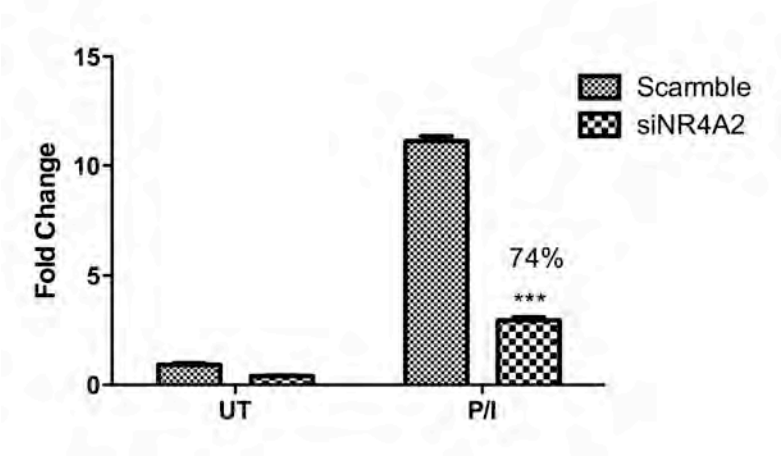
Figure 9. Adenosine A_{2A} receptors modulated NR4A2 expression and chemokine release (A) Reduction of P/I-mediated NR4A2 transcriptional induction by CGS-21680 (1 μM). The data were normalized to the housekeeping gene (β-actin gene). Fold changes are calculated against untreated controls (B) Cells were silenced by A_{2A}AR siRNA or pretreated with CGS-21680 (1 μM) for 15min then stimulated as indicated in the figures. GAPDH is included as an internal control (C) and (D) The culture supernatants were collected IL-4 concentrations determined by ELISA. Values represent the mean of three independent experiments ±SEM (E) Co-transfected of LAD-2 with 3xNBRE or wt-κB and pRL-TK vector for 24 h. Cells were treated as indicated. Firefly (NBRE or wt-κB)/renilla ratio was shown. Means and ±SEM of the firefly/renilla ratios of at least three independent experiments are shown

Supplementary Figure 1



Cell viability was tested by Alamar blue assay，none of these treatments affected cell viability. LAD-2 cells were seeded in 96-well plates (45,000 cells/well/90 µl). Different stimuli were added as indicated in the figure, after 2 h or 20 h, 10 µl Alamar blue (Serotec) was applied for each well. After 4 h, fluorescence was monitored at 530-nm excitation and 590-nm emission wavelength (LS55 luminescence spectrometer, Perkin-Elmer).

Supplementary Figure 2



Analysis of efficiency of NR4A2 silencing. Transfection of LAD-2 cells with NR4A2 siRNA specifically downregules NR4A2 expression induced by P/I. Scramble siRNA was included as a control 74.2%,

Supplementary Figure 3

GENE	P/I Scramble RNA	P/I siNR4A2
CCL1	159.8*	151.7
CCL18	5.8	6.6
CCL2	145.6	136.7
CCL23	4.6	2.1
CCL3	269.7	74.1
CCL4	980.9	813.5
CCL5	9.0	5.2
CCL7	2914.4	2242.7
CCRL2	2.2	2.2
CLCF1	53.3	34.7
CSF2	310.6	288.6
CXCL12	6.5	22.3
CXCR4	45.8	27.3
HIF1A	2.0	1.4
IL1A	7.1	5.1
IL1B	12.3	7.4
IL1RAP	4.2	2.0
IL4	951.5	128.4
IL8	44824.5	13070.3

P/I induced NR4A2-dependent Genes by 96-well RT-PCR screening. LAD-2 cells were transfected with scramble or NR4A2 siRNA for 48 h. Total RNA were harvested after 6 h with or without PMA/ionomycin (P/I, PMA: 25 nM, ionomycin: 0.1 μM). Fold changes are calculated against untreated. * Fold changes

5- Discussion and Conclusions

Mast cells are granulated immune cells that often act as the first line of immune defense. They have been traditionally associated to immediate-type hypersensitivity reactions through the release of preformed inflammatory mediators. However, today it is well accepted that mast cells exert various other physiological functions. Dependent on the model of activation, mast cells can release distinct mediator profiles thereby enabling the initiation, maintenance and modulation of both innate and acquired immune responses [173-175]. To serve this function, mast cells are equipped with a multitude of receptors serving directly or indirectly as sensors of external stimuli. The engagement of activating and inhibitory cell-surface receptors, as well as the intensity and duration of these signals determines the activation state of mast cells. In response to these stimuli, gene expression patterns of inflammatory mediators are altered according to the sum of positive and negative signaling events, thereby affecting the course of inflammation [176]. The potency of adenosine as a modulator of mast cell function is well recognized, and ARs have been the center of much interest as therapeutic targets for asthma and allergy [65, 115]. Although *in vitro* studies have suggested roles for each AR subtype on mast cells, the transcriptional effectors downstream of ARs activation as well as the biological consequences of their activation have remained largely unknown.

In the first part of this study, we employed the extensively characterized HMC-1 human mast cell line to investigate downstream AR signaling. By combining selective AR targeting and transcriptomics, we were able to establish a novel link between AR activation and NR4A orphan receptors. Interestingly, our genome-wide screening and RT-PCR confirmatory experiments revealed a rapid and strong upregulation of NR4A2 and NR4A3 (but not NR4A1) upon treatment with the adenosine analogue NECA, suggesting that these transcription factors may act as important transcriptional mediators of inflammatory signals. Further, we showed that selective activation of the A_{2A}AR negatively regulates the induction of these factors, demonstrating divergent effects between the AR subtypes on the induction of these orphan receptors. On one hand, the activation of pro-inflammatory ARs (i.e. A_{2B} and A_3ARs) act as an amplification signal resulting in not only very high levels of NR4A2 and NR4A3 mRNA (but not of NR4A1), and also induced NR4A2 protein accumulation and NBRE transcriptional activity. On the other, this upregulation is multiplied by blocking the anti-inflammatory A_{2A}AR. This results support previous reports that indicated that NR4As participate in inflammation related conditions, such as synovitis [191, and proposes one mechanism of A_{2A}AR-mediated anti-inflammatory effect for such conditions adenosine's effect on this group of transcription factors could have broad biological implications.

Activation of mast cells (for example by the high affinity IgE receptor) requires the activation of receptor-proximal tyrosine kinases, mobilization of internal Ca^{2+} and the formation of signaling complexes coordinated by adaptor proteins [177]. PI3K is a central player in mast cell activation that signals to regulatory enzymes such as PKC, PLC as well as PLD among others. Thereby Ca^{2+} ultimately regulates mast cell degranulation, the biosynthesis of inflammatory molecules such as arachidonic acid metabolites, as well as cytokine transcription [178]. Adenosine receptor activation has traditionally been linked to stimulation or inhibition of the AC; A_{2A}AR and A_{2B}AR activation lead to increased cAMP levels that in turn activate the canonical PKA pathway and the exchange protein directly activated by cAMP (Epac) [179],while A_1AR and A_3AR activation leads to cAMP decrease [75]. In addition, AR signaling in mast cells has also been linked to PLC and Ca^{2+} mobilization (A_{2B}AR and A_3AR), PI3K (A_3AR), as well as PKC and MAP kinases (A_1, A_{2A} and A_3AR) [65, 180]. Here, we analyzed the signaling pathways involved in AR-induced NR4A upregulation and we found that AR-mediated NR4A2 and NR4A3 upregulation in HMC-1 did not involve PKA, PI3K nor p38. Instead, PKC and MEK kinase inhibition could partially revert the induction of these factors and, moreover, the activity of these kinases correlated with ERK1/2 phosphorylation. Interestingly, some studies have also shown the involvement of ERKs and Ca^{2+} signaling pathways in adenosine signaling [181], but downstream targets of ERK upon AR activation had not been identified. The results presented here suggest that activation of ERK1/2 kinases downstream of PKC mediates NR4As induction by AR. Remarkably, A_{2A}AR can counterbalance NECA-induced ERK1/2 phosphorylation, correlating with its modulatory effect on NR4A2 and NR4A3 induction.

NR4As also influence the function of other inflammation-associated transcription factors. For example, NR4A1 and NR4A2 form heterodimers with retinoic acid receptor and can influence retinoid signaling [182]. Therefore, AR activation could affect the number of NR4A-containing complexes. In addition, NR4A receptors can also crosstalk with other transcriptional factors and influence their activity without necessarily interacting with them. For example, NR4A receptors and the estrogen-related receptor NR3B mutually repress each other's transcriptional activity [183]. Similarly, NR4A1 has been shown to negatively cross-talk with NF-κB [184]. As a consequence, by virtue of the induction of remarkably high levels of functional NR4A2 and NR4A3, adenosine is likely profoundly affect the expression of large sets of genes both directly (NBRE-responsive transcripts) and indirectly, by affecting the nature of transcriptional complexes and/or crosstalking with other transcriptional factors. Conversely, A_{2A}AR activation could limit the availability of NR4A2 and NR4A3 for heterodimerization or cross-talk with other transcriptional factors.

The focus in the second part of this thesis was on investigating whether the inhibitory effect of A_{2A}AR on NR4A2 induction was preserved to other mast cell activating stimuli. For this we employed the more mature LAD-2 cell line, which possess several characteristics of fully differentiated mast cells [171]. To achieve this goal, we first characterized the signaling mechanisms triggered by the concomitant treatment

of mast cells with PMA and ionomycin (P/I). Our results showed not only the upregulation of NR4A2, but also established a previously undescribed pattern of post-translational modifications of this transcription factor. Ubiquitination of NR4A2 and the associated re-localization and downstream effects observed imply an additional level of regulation of this transcription factor, supplementing the well-characterized regulation at the transcriptional level of this ligand-independent, constitutively active factors. Ubiquitination can be an essential step for rapid degradation of a protein with short half-life and can therefore regulate the transcriptional activity of transcriptional factors [185]. The results presented here supports this this hypothesis, since proteasome inhibition and inhibition of ubiquitinating enzyme I (UBE1, which catalyzes the first step in the ubiquitination process) effectively resulted in accumulation of ubiquitinated NR4A2 species. This was confirmed by immunoprecipitation of NR4A2, which specifically cross-reacted with ubiquitin antibodies. Further, we established that NR4A2 ubiquitination was phosphorylation-dependent, suggesting a link between kinase signaling cascades and NR4A2 modifications. In support of this hypothesis, NR4A1 has been shown to be a downstream phosphorylation target of JNK kinases, which resulted in its ubiquitination and degradation [28]. Our experiments in fact show that both upregulation and post-translational modifications of NR4A2 are at least partially dependent on PKC and MEK kinases, although it remains unclear whether both events are equally dependent on these kinases, or whether the negative effect of PKC/MEK inhibition on NR4A2 post-translational modifications is a consequence of their effect on NR4A2 abundance. Previous studies showed that phosphorylation of NR4A1 regulates nuclear export, and cytoplasmic and mitochondrial localization [186]. Here we employed a combination of approaches, including cell fractionation and western blotting, confocal microscopy to establish that ubiquitinated NR4A2, unlike phosphorylated NR4A1, locates in to ER, endosomes and lysosomes, and that these cytoplasmic signals can be stabilized by proteasome inhibition.

The second major finding of this study was the identification of NR4A2 as a central factor in IL-4 release upon P/I stimulation, supporting a pro-inflammatory role for the NR4A2 on human mast cells in response to activating stimuli. Furthermore, ubiquitination of NR4A2 seemed to play a role in IL-4 induction, with MG132 having a *biphasic* effect on IL-4 release: treatment with this proteasome inhibitor further increased its release in an early time point (6 h), while it dramatically decreased IL-4 secretion at late phase (24 h). By a combination of silencing and NF-κB and NR4A reporter assay approaches, we showed that the early IL-4 response (6 h) after P/I treatment depends on NR4A2, while NF-κB appears to be the main driver of the late responses. Several studies have investigated the cross-talk between NR4As and NF-κB. Both NR4A1 and NR4A2 have been proposed as a downstream target of NF-κB in response to inflammatory mediators [42, 187]. More recently, NR4A2 has been shown to contribute with NF-κB towards the expression of IL-8 [188]. The data presented further expands our understanding on the cross-talk between NR4A2 and NF-κB. Further, we show that the accumulation of ubiquitinated NR4A2 in the ER

leads to the induction of CHOP, a surrogate of ER-stress [189, 190]. ER stress can lead to NF-κB activation by at least 3 different pathways: through TRAF2 dependent IKK phosphorylation; through PERK-induced phosphorylation of eIF2α**,** which decreases IκB protein by repression of IκB translation, leading to interference with the export of nuclear NF-κB to the cytoplasm; or through ATF6, which causes activation of NF-κB via the Akt pathway [191]. Further studies will be required to establish the relevance of NR4A2-mediated ER stress in late NF-κB activation and IL-4 induction.

IL-4 is a prototypic immunoregulatory cytokine responsible for initiating allergic responses and it essential is uniquely required to polarize naïve helper cells (Th0 cells) to a type 2 phenotype, characterized by secretion of IL-4 itself, plus IL-5, IL-9, IL-10, and IL-13. IL-4 also induces B cells to switch their immunoglobulin production to IgE secretion. Mast cells and basophils readily produce IL-4 after crosslinking of their FcεRI receptors [192]. Here we present a novel link between P/I activation of mast cells and IL-4 release, and we show that the late release of IL-4 depends on NR4A2-induced NF-κB activity, suggesting that although proteasome inhibition enhances early IL-4 release, it could potentially translate in downregulation of sustained, IL-4 mediated allergic responses in vivo.

Finally we addressed the question on how A_{2A}AR activation could influence NR4A2 and NF-κB dependent IL-4 induction, and showed that the concomitant activation of this AR subtype has a broad modulatory effect on at all levels tested, which resulted in a significant decrease of IL-4 release, suggesting that A_{2A}AR activation could modulate influence the development of allergic Th2 responses, in support of previous studies that observed that A_{2A}AR activation successfully suppressed asthmatic inflammation in animal models [97] .

6- Additional publications

6.1 Adenosine signaling in equine recurrent airway obstruction

Recurrent airway obstruction (RAO, also known as COPD or heaves) and inflammatory airway disease (IAD) in the lower airway are common diseases of horses characterized by airway narrowing, airway hyper-reactivity, chronic lower airway inflammation, reversible airflow obstruction and respiratory difficulty [193, 194]. RAO appears to be happened in older horses, while IAD affects most in younger horses [195] and the prevalence of RAO can be as high as 50%, ranging from subclinical to overtly manifested cases. It has been shown that RAO involves hypersensitivity to inhaled antigens, although multiple theories exist regarding exactly why it occurs [193]. Several lines of evidence suggest that adenosine plays a central role in the pathogenesis of lower airway inflammation (in chapter 2.2.4). Based on the potential of AR modulation in chronic airway disease, this project was aimed at identifying and characterizing adenosinergic signaling in equine airways.

Here, 2 publications are presented. In the first one, the adenosine content in bronchoalveolar lavage fluid samples from healthy and airway-compromised equines was investigated. In the second one the effects of AR signaling in the equine airways is established though *ex vivo* stimulation of bronchoalveolar lavage-recovered cells.

In summary, the results of this research project confirms the presence of measurable adenosine levels in lower airways in the horse, which are increased during inflammatory processes, and also the presence of functional adenosine signaling axis which may constitute the basis for more specific research of AR-based therapies for equine RAO and possibly for other chronic airway diseases in the horse.

6.2 Increased adenosine concentration in bronchoalveolar lavage fluid of horses with lower airway inflammation

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Short Communication

Increased adenosine concentration in bronchoalveolar lavage fluid of horses with lower airway inflammation

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ABSTRACT

Several reports have suggested a role for adenosine in the pathogenesis of chronic airway conditions and this has led to new therapeutic strategies to limit airway inflammation. In this study, detectable levels of adenosine in bronchoalveolar lavage (BAL) samples from 11 horses with non-infectious lower-airway inflammation and 14 healthy controls are reported, with significantly higher values in horses with airway inflammation. Although these increased levels did not correlate with changes in neutrophil percentage in BAL, a positive association between adenosine levels and signs of lower airway inflammation (clinical score) was observed. These novel findings support the hypothesis that adenosine may contribute to bronchoconstriction and also act as a pro-inflammatory mediator in the bronchoalveolar milieu of horses with airway inflammation. Further investigation of this axis could lead to new approaches for the treatment of highly prevalent lower airway inflammatory conditions in the horse.

Adenosine is an endogenous nucleoside that accumulates in bronchoalveolar lavage (BAL) in patients with asthma (Driver et al., 1993). Administration of adenosine by inhalation induces bronchoconstriction of asthmatic (but not of normal) airways. Recently, adenosine 5′-monophosphate has been shown to have a similar effect on cats affected with chronic airway disease (Hirt et al., 2011). In addition, adenosine modulates the course of inflammation through the release of cytokines and chemokines from various cell types in the airways (Caruso et al., 2009). Therefore, strategies based on adenosine receptor ligands are currently being developed aimed at controlling adenosine-mediated inflammatory signalling (Dip, 2009).

Persistent inflammation is a central feature of chronic non-infectious airway conditions, such as recurrent airway obstruction (RAO) and inflammatory airway disease (IAD). RAO is characterised by bronchospasm, mucus secretion, airway wall thickening and deterioration of lung function, and affects older individuals. IAD presents clinical and cytological similarities with RAO, but usually affects younger horses and clinical signs are mild (Couetil et al., 2007).

In the current study, we wanted to determine whether increased adenosine levels are associated with lower airway inflammation in the horses. A total of 11 adult horses (three mares and eight geldings; age range 7–17 years, median 9.5 years) with mild to moderate signs of airway inflammation were included in the study. Lower airway inflammatory disease (either IAD or RAO) was diagnosed based on clinical signs, history, mucus (amount, quality and cellularity) and cytological examination of BAL samples. Horses medicated within 28 days prior to the examination and those with concomitant signs of infectious or systemic disease were excluded from the study. In addition, 14 healthy controls (four mares and 10 geldings; age range 5–22 years, median 11 years) with no history of airway inflammation or clinical signs of respiratory disease and normal BAL cytology were employed as a control group.

Each horse was clinically examined and given a weighted score, using a scale with range 0–25 as described by Tesarowski et al. (1996; see Appendix 1, Supplementary data). Two individuals performed all clinical observations. All healthy controls had clinical scores of 2 or lower (range 0–2, median 0.5) while horses with airway inflammation had higher scores (range 0–8, median 4), corresponding to moderate clinical sings (horses with experimentally induced RAO had mean scores of approximately 12; Tesarowski et al., 1996).

The BAL and cytological analysis was performed as previously described (Zhang et al., 2011). BAL-samples were collected in vials containing 0.1 μM iodotubericidin and 10 μM of erythro-9-(2-hydroxy-3-nonyl)adenine (Sigma), centrifuged, and cell-free fractions immediately frozen. All samples were analysed by a specific spectrometry-based assay at a later time point (Ren et al.,

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Table 1
BAL cytology, clinical score and adenosine concentration from horses with airway inflammation (15–25) and healthy controls (1–14).

Horse	Sex	Age	Neutrophils (%) [RV ⩽ 5%][a]	Lymphocytes (%) [RV 30–50%]	Macrophages (%) [RV 40–60%]	Eosinophils (%) [RV ⩽ 0.5%]	Basophils (%) [RV ⩽ 2%]	Clinical score	Uncorrected adenosine concentration (pg/μL)	Corrected adenosine concentration (pg/μL)
1	M	10	1.3	46.6	52.1	0.0	0.0	0	40.5	51.1
2	F	10	1.3	45.9	52.1	0.7	0.0	1	43.1	67.9
3	M	17	1.9	52.2	45.4	0.0	0.5	0	46.9	25
4	M	5	5.7	56.3	36.8	0.0	1.2	1	108.6	140.1
5	F	15	6.1	69.3	24.2	0.0	0.4	1	52.9	32.8
6	M	5	0.8	48.9	46.8	0.0	3.5	0	38.7	64
7	F	8	2.1	41.6	55.2	0.0	1.1	0	44.1	41
8	M	16	3.4	62.4	33.3	0.0	0.9	1	29.0	20.9
9	F	9	1.9	46.9	48.3	0.5	2.4	0	127.7	237.5
10	M	5	3.5	57.9	38.2	0.0	0.4	0	31.2	27.1
11	M	5	1.2	36.0	62.2	0.0	0.6	0	101.1	174.3
12	M	10	6.0	39.0	53.4	0.0	1.6	2	17.0	20.6
13	M	13	3.5	32.2	60.4	0.0	0.0	2	17.2	23.8
14	M	9	0.5	60.5	37.0	0.5	1.5	1	191.8	180.1
Average		9.8	2.8	49.6	46	0.1	1	0.6	63.57	79.02
Range		5–16	0.5–6.1	32.2–69.3	24.2–62.2	0–0.7	0–3.5	0–2	17.0–191.8	20.6–237.5
15[b]	M	6	5.7	61.9	31.5	0.0	1.0	1	85.4	71.7
16	M	12	5.6	38.1	53.8	0.2	2.5	8	215.8	231.4
17	M	7	10.4	53.9	35.7	0.0	0.7	4	26.4	21.5
18	M	7	7.3	61.4	31.3	0.1	1.0	0	57.8	64.9
19	F	15	4.1	25.2	67.4	0.0	0.5	5	34.7	50
20	M	5	4.5	46.8	47.8	0.0	0.2	4	377.3	315.1
21	F	11	31.9	20.2	43.7	0.5	0.0	3	112.8	101
22	F	13	12.4	59.7	27.2	0.0	0.7	8	316.3	367.7
23	M	8	16.0	48.1	31.4	0.0	4.5	8	201.7	217.1
24	M	22	76.3	11.8	11.4	0.1	0.5	8	352.0	228.9
25	M	13	50.0	23.9	25.7	0.0	0.4	2	103.5	131.1
Average		10.8	20.7	41.1	37.1	0.1	1.1	4.6	171.23	163.68
Range		5–22	4.1–76.3	11.8–61.9	11.4–67.4	0–0.5	0–4.5	0–8	26.4–377.3	21.5–367.7

RV, reference value.
[a] Neutrophil counts higher than 5% were considered indicative of airway inflammation. However, values between 4% and 6% were interpreted in relation to other diagnostic criteria.
[b] Lower airway inflammation was diagnosed on the basis of medical history and decreased exercise tolerance.

2008). Briefly, a Zorbax eclipse XBD-C-18 column (Agilent) was used with a TSQ Quantum-Ultra system and the HSE source (Thermo Electron). The mobile phase consisted of linear gradient changes of 0.1% formic acid in water and 0.1% formic acid in methanol.

The use of dilutional markers has been investigated in the horse, but remains a matter of controversy (Kirschvink et al., 2001). To avoid dilutional artefact, urea concentrations were determined (QuantiChrom assay, BioAssay Systems) in both in BAL and serum samples that had been collected simultaneously. The ratio between urea concentrations served as an indicator of epithelial lining fluid dilution and was employed for normalisation (corrected adenosine concentration).

The BAL cytology, clinical score, and uncorrected and corrected adenosine concentrations are summarised in Table 1. Interestingly, these data suggested a limited dilution effect on BAL samples when considered collectively, despite dilution differences of individual samples. Uncorrected adenosine values were employed for further analysis, following the recommendation of the European Respiratory Society that acellular BAL components should be expressed as concentration per volume recovered.[1]

Each BAL sample revealed adenosine levels above the limit of detection (LOD: 0.02 pg/μL BAL). Furthermore, adenosine concentration was significantly higher in horses with airway conditions (range 26.4–377.3 pg/μL, median 112.8 pg/μL), compared to healthy controls (range 17–191 pg/μL, median 43.6 pg/μL), which suggested a link between lower airway inflammation and adenosine in this species (Fig. 1).

The association between adenosine concentration in BAL and clinical findings was then assessed quantitatively, independently of disease state. Fig. 2A shows the significant correlation (Spearman) between adenosine concentration and clinical score. A similar correlation was observed when only horses with airway inflammation were considered.

Since the assessment of BAL cytological alterations (neutrophils relative counts) remains a major diagnostic tool for lower airway disease, the association between neutrophil percentage and adenosine concentration was investigated. In contrast to clinical score, a correlation between these two variables could not be established (Fig. 2B). Yet this observation should be interpreted with care because only three samples with neutrophil count indicative of moderate to severe airway inflammation (>20%) were tested. Analysis of a larger set of samples with high percentage of neutrophils (i.e. acute RAO phase) would be required to clarify the relationship between these two parameters.

The presence of increased adenosine concentrations in horses with airway inflammation suggested that adenosine could represent a marker of inflammation. However, in view of the partial overlap in the values of both groups of horses analysed, assessment of adenosine levels in BAL did not seem to be suitable as the sole indicator of disease, but should be interpreted in combination with other diagnostic parameters (i.e. clinical signs).

[1] See: http://www.ers-education.org/pages/default.aspx?id=335&idBrowse=37508&det=1.

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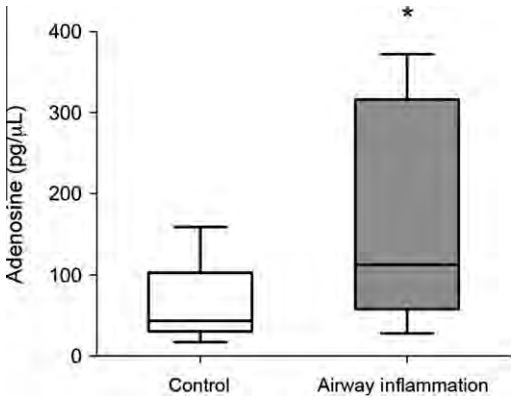

Fig. 1. Uncorrected adenosine concentration in BAL fluid samples in horses with airway inflammation and controls. * $P \leqslant 0.05$, compared to the control group (Mann–Whitney *U*-test).

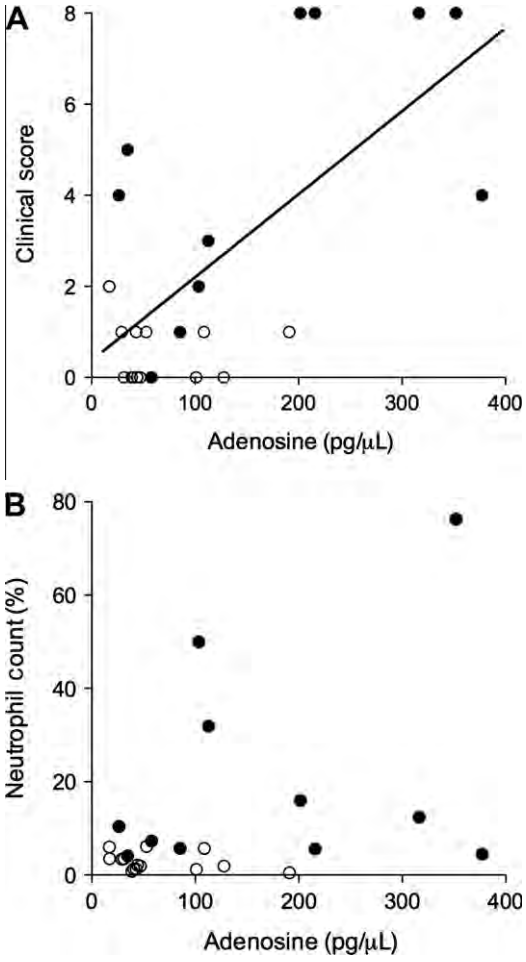

Fig. 2. (A) Positive correlation between adenosine concentration and clinical score for all 25 horses included in the study (Spearman *r* correlation coefficient = 0.36, $P \leqslant 0.05$; slope: 0.018, $P \leqslant 0.05$). A similar correlation is observed when considering only horses with airway inflammation (Spearman *r* correlation coefficient = 0.53, $P \leqslant 0.05$). (B) Association between adenosine concentration and neutrophil count. No significant correlation was observed; (●), horses diagnosed with lower airway inflammation; (○), healthy controls.

Two initial reports suggested the presence of an adenosine-signalling axis in equine airways. One early study indicated that adenosine influences contractility in equine tracheal smooth muscle (Norris and Eyre, 1982). Our own research group then recently showed enhanced interleukin (IL)-6 transcriptional responses to adenosine receptor ligands in BAL cells from horses with lower airway inflammation (Zhang et al., 2011). The increased adenosine levels reported in the current study therefore supported the hypothesis that this nucleoside both contributes to bronchoconstriction and acts as a pro-inflammatory mediator in the bronchoalveolar milieu of horses with airway inflammation. Further characterisation of this inflammatory signalling axis could potentially lead to new approaches for the treatment of highly prevalent lower airway inflammatory conditions, such as RAO and IAD.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tvjl.2011.11.012.

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6.3 Enhanced IL-6 transcriptional response to adenosine receptor ligands in horses with lower airway inflammation

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Enhanced IL-6 transcriptional response to adenosine receptor ligands in horses with lower airway inflammation

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Summary

Reasons for performing study: Accumulation of extracellular adenosine has been closely associated with human asthmatic responses. However, the relevance of adenosine signalling in equine airways has not previously been investigated.
Objectives: To determine the expression of adenosine receptors (AR) in bronchoalveolar lavage (BAL) cells and assess the reactivity of these cells to AR ligands *ex vivo*, employing IL-6 as readout of adenosinergic inflammatory signalling.
Methods: Eight horses with varying degrees of lower airway inflammation and 10 healthy controls were analysed. Expression of AR-subtypes in each BAL sample was determined by quantitative RT-PCR and compared to that in 13 other tissues. Bronchoalveolar lavage cells were stimulated either with the adenosine analogue NECA, CGS-21680 (A_{2A}AR selective agonist) or with a combination of NECA and SCH-58261 (A_{2A}AR antagonist) and IL-6 expression assessed.
Results: Bronchoalveolar lavage cells predominantly expressed A_{2B}AR, with lower A_{2A}AR levels and marginal A_3AR expression; A_1AR was not detected. This pattern was similar to that of PBMCs but different from the other tissues tested. No significant differences in AR expression in BAL cells from both groups were detected, although a trend for decreased A_{2B}AR in airway-compromised horses was observed. Treatment of BAL cells with the nonselective agonist NECA upregulated IL-6 expression in cells from airway-compromised horses, but levels remained unchanged in control animals. Furthermore, blockage of A_{2A}AR with SCH-58261 enhanced IL-6 mRNA induction by NECA in both groups, with higher levels in airway-compromised horses; the amplitude of this response correlated with neutrophil count.
Conclusions: These results demonstrate the presence of an adenosine/IL-6 inflammatory axis in the bronchoalveolar milieu of airway-compromised horses. While A_{2B}AR is the predominant proinflammatory AR subtype expressed, A_{2A}AR appears to modulate inflammatory signalling (IL-6 expression) by adenosine.
Potential relevance: This study supports selective AR targeting as a potential therapeutic approach for the modulation of inflammation in the equine lower respiratory tract.

Keywords: horse; airway inflammation; adenosine; IL-6; adenosine receptor

Introduction

Chronic inflammatory airway diseases are remarkably prevalent in the horse. Recurrent airway obstruction (RAO) and inflammatory airway disease (IAD) are 2 such conditions that share a number of clinical, cytological and functional similarities. Recurrent airway obstruction is characterised by bronchospasm, mucus secretion, airway hyperreactivity and airway wall thickening together with deterioration of lung function and affects older individuals, while IAD-affected horses are usually younger with clinically milder signs (Couetil *et al*. 2007).

Adenosine is an endogenous byproduct of ATP metabolism normally present at low concentrations in the extracellular space. Its levels are greatly increased under metabolically stressful conditions such as tissue injury, hypoxia and acute or chronic inflammation, reaching local concentrations of up to 30 μmol/l, a 150-fold increase over basal levels (Van Belle *et al*. 1987). This nucleoside activates 4 types of G-coupled adenosine receptors (ARs), which are expressed on various inflammatory and stromal cells: A_1, A_{2A}, A_{2B} and A_3. ARs have well characterised anti-inflammatory and wound healing activities, demonstrating the importance of these signalling pathways in tissue protection (Hasko *et al*. 2008). In some situations, however, adenosine generation and AR activation have proinflammatory activities that amplify tissue injury (Blackburn 2003). These observations have driven research efforts to develop selective agonists or antagonists for AR subtypes for use in novel therapies for chronic inflammatory conditions.

There is growing evidence that adenosine plays an important role in respiratory disorders in the lower airways. Increased amounts of adenosine are found in bronchoalveolar lavage and exhaled breath condensate of human patients with asthma (Driver *et al*. 1993; Huszar *et al*. 2002) and, when administered by inhalation, adenosine was shown to be a powerful bronchoconstrictor in asthmatics but not in healthy subjects (Cushley *et al*. 1983). Furthermore, blockade of adenosine reuptake by dipyridamole increased the bronchoconstrictor response to adenosine in asthma indicating that its accumulation is closely associated with the asthmatic airway response (Cushley *et al*. 1985).

Recent studies have investigated the adenosine pathway in the horse. The affinity of several ligands to AR subtypes has been studied in horse neutrophils (Sun *et al*. 2007). Equine A_{2A} and A_3ARs have been cloned in this species (Brandon *et al*. 2006a,b). Furthermore, activation of A_{2A}AR in equine monocytes has been shown to inhibit lipopolysaccharide-induced TNF-α production, suggesting a therapeutic potential for AR modulation in inflammation (Sun *et al*. 2008a). However, the relevance of adenosine signalling on the regulation of inflammatory cytokines in the equine respiratory tract has not previously been examined.

Here, we employed an *ex vivo* approach to assess the role of adenosine in the physiopathology of lower airway inflammation in the horse. First, we established the expression profile of ARs in BAL cells and compared it to cerebellum, cortex, myocardium, aorta, spleen, stomach, jejunum, caecum, kidney, adrenal gland, eye, fat and peripheral blood mononuclear cells (PBMCs). Second, we determined that AR levels do not differ significantly between compromised and control animals. Third, we investigated the reactivity of BAL-recovered cells from horses with lower airway inflammation and healthy controls to AR ligands and determined that nonselective pharmacological activation of ARs leads to increased expression of the pro-inflammatory cytokine IL-6 in cells from horses with lower airway inflammation but not from control animals. Finally, we observed that selective blockage of A_{2A}AR further increases IL-6 transcription, leading to remarkably high levels in horses with lower airway inflammation. These data suggest that adenosine signalling is a relevant inflammatory mechanism during lung inflammatory conditions and that A_{2A}AR acts as an endogenous modulator of inflammatory signalling in the equine airway.

Materials and methods

Horses

Eight adult horses (2 mares and 6 geldings; age range 5–18 years, median = 13 years; weight range 450–516 kg) were presented with varying degrees

TABLE 1: Primer sequences and expected product lengths of the genes analysed

Gene	GenBank accession No.	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product length (bp)
A_1AR	XM_001496410	TCAACATCGGGCCACGGACC	CAGGTTGTTCCAGCCAAACAGAG	242
A_{2A}AR	NM_001081897	CGCGAGTTCCGCCACACCTT	CCTGCTCTGCATCGCTGCCA	124
A_{2B}AR	XM_001918390	TCACDCAGAGCTCCATCTTC	CAAAGGCAAGGACCCAGAGG	141
A_3AR	AY_011243	AGGGACACAGGAAGCCAGCTCA	GGAAGCCCTGCAGCTTCTGTTCC	213
IL-6	NM_001082496	ATGAGTGGCTGAAGAACACAACAAC	AGGAATGCCCATGAACTACAACAAT	131
GAPDH	NM_001163856	TGGCATGGCCTTCCGTGTCC	GCCCTCCGATGCCTGCTTCAC	118

of lower airway inflammation at the Equine Clinic at the University of Zurich. Clinical signs included exercise intolerance, coughing, nasal discharge and the horses were diagnosed with lower airway inflammatory disease (either IAD or RAO) on the basis of history, clinical findings, mucus and cytological examination of BAL samples.

Ten healthy horses with no history of airway inflammation or clinical signs of respiratory disease and normal BAL cytology were employed as a control group (7 mares and 3 geldings; age range 8–17 years, median = 9 years; weight range 480–605 kg). These horses belonged to the same stud, were vaccinated and dewormed regularly, stabled in the same barn and fed hay and grain twice a day. Sample collection of control horses was approved by the veterinary district authorities; anaylsis of samples obtained for diagnostic purposes did not require approval.

Bronchoalveolar lavage and differential cytology

Horses were sedated with a combination of xylazine (0.1 mg/kg bwt i.v.)[1] and butorphanol (0.02 mg/kg bwt i.v.)[2]. A fibroscope was introduced into a nostril, directed into the trachea and advanced until it wedged in a bronchus. During the advancement of the bronchoscope the bronchial surface was continuously anaesthetised with 50 ml of a solution containing 0.4% lidocaine and 0.9% sodium chloride injected through a sterile catheter passed through the biopsy channel. Two-hundred-and-fifty ml of prewarmed (37°C) sterile physiological saline solution were then infused into the bronchus and immediately reaspirated into a sterile glass flask kept on ice.

Bronchoalveolar fluid was centrifuged at 200 ***g*** for 10 min and rinsed x1 with PBS. A cytospin slide was prepared and stained with May-Grünwald Giemsa for cytological analysis. At least 400 cells from each lavage sample were counted.

Cell culture and *ex vivo* stimulation

The remaining cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% FBS[3], 0.1 u/ml penicillin and 0.1 mg/ml streptomycin[3] at a concentration of 2×10^6/ml, 10^6 cells were seeded in each well of a 24 well plate and cultured overnight at 37°C under humidified air containing 5% CO_2. The following day cells were treated for either 3 h with the AR ligands 5′-N-ethylcarboxamidoadenosine (NECA, 10 μmol/l), 2-p-(2-carboxyethyl)phen ethylamino-NECA (CGS-21680, 0.1 μmol/l) or the combination of NECA and 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine (SCH-58261, 1 μmol/l). All chemicals were from Sigma[4]. The use of lidocaine as a local anesthetic during the BAL procedure did not contribute to any observed effect as there was an extensive rest period before the cells were used.

RNA extraction and cDNA synthesis

Total RNA was isolated and cDNA synthesised as described (Franchini *et al*. 2000). Briefly, RNA was isolated using the total RNA isolation kit, which includes a DNAse treatment step[5]. RNA concentration was measured by optical density at 260 nm and RNA quality assessed by 260/280 absorvance ratio and cDNA synthesised with the cDNA reverse transcription kit[6] following the manufacturer's instructions. Briefly, 0.5 μg of total RNA was mixed with 1 μl of random primers, 2 μl of reverse transcription buffer and 0.8 μl dNTP mix, 1 μl multiScribe reverse transcriptase and 1 μl RNA inhibitor to a final volume of 20 μl. The mix was incubated at 25°C for 10 min and at 37°C for 120 min and then heated at 85°C for 5 s. The reactions were stored at -20°C until used.

Samples for adenosine receptor expression profiling

Immediate *post mortem* samples were obtained from one healthy horse. 200 mg of cerebellum, cortex, myocardium (left ventricle), aorta, spleen parenchyma, jejunum and caecum (submucosa and muscular), kidney (cortex), adrenal (cortex and medulla), eye (conjunctiva) and retroperitoneal fat were mechanically disrupted in a 3 ml Dounce homogeniser in lysis buffer[5]. Additionally, 10 ml of whole blood were obtained from this animal and PBMCs isolated employing a Ficoll reagent, following the manufacturer's instructions[7]. Ribonucleic acid from each tissue and PBMCs was extracted and cDNA synthesised as described above.

Selection of primers for expression analysis

The ARs primers for RT-PCR were designed based on the complete (A_{2A}), predicted (A_1, A_{2B}) or partial cd sequences (A_3) (GenBank accession numbers: NM_001081897, XM_001496410, XM_001918390 and AY_011243, respectively). Primers were selected for RT-PCR performance and absence of artifacts. The specificity of the amplified product was confirmed by sequencing. Amplification efficiency was calculated based on the CT values obtained from a serial dilution of a positive control cDNA (90–110%). Sequences for A_1, A_{2A}, A_{2B} and A_3-ARs, IL-6 and GAPDH primers and their expected product lengths are listed in Table 1. PCR reactions with these primers yielded single bands of the expected sizes. No unspecific secondary primer bands were observed (Supplementary Fig S1).

Quantitative real-time polymerase chain reaction (RT-PCR)

One μg cDNA was amplified in a 20 μl PCR reaction containing 10 μl of SYBR green supermix[8] and 10 μmol/l of forward and reverse primers. The PCR was performed in the iCycle IQ multicolour real time PCR detection system[8] with an initial incubation step at 95°C for 10 min to activate the enzyme and 45 cycles of amplification, including a denaturation at 95°C for 15 s and a 1 min extension at 60°C. Samples without cDNA were always included in the amplification reactions to check for contamination. All reactions were performed in triplicates. Fold changes in the expression of each gene was calculated using the 2-ΔΔCT method (Livak and Schmittgen 2001), with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript as an internal standard. Relative AR and IL-6 levels in resting cells are expressed as the ratio of the CT value for the gene of interest/CT for GAPDH. Mean GAPDH CT values for airway and control horses are 19.98 and 20.35 (difference not significant), with coefficients of variation are 3.68 and 3.65%, respectively. Finalised RT-PCR reactions were loaded in a 2% agarose gel containing 0.1 mg/ml etidium bromide for visualisation of DNA and subjected to electrophoresis.

Data analysis

Statistical analysis was performed using the PASW Statistics software[9]. Data were analysed by nonparametric tests (Mann-Whitney *U* test, Spearman correlation coefficient) or with the *t* test with Welch correction when values showed normal distribution (A_{2B}AR expression). Differences between groups were considered significant when P values were less than 0.05.

Results

Cytological characterisation of BAL samples

Control horses had cytological values within physiological range (neutrophils <5%, lymphocytes 30% to 60%, macrophages 40–70%,

TABLE 2: Cytological findings in BAL cells from healthy (1–10) and airway-compromised horses (11–18)

Horse	Sex	Age	Neutrophils (%)	Lymphocytes (%)	Macrophages (%)	Eosinophils (%)	Basophils (%)
1	F	10	1.3	45.9	52.1	0.7	0
2	M	17	1.9	52.2	45.4	0	0.5
3	M	5	5.7	56.3	36.8	0	1.2
4	F	15	6.1	69.3	24.2	0	0.4
5	M	5	0.8	48.9	46.8	0	3.5
6	F	8	2.1	41.6	55.2	0	1.1
7	M	16	3.4	62.4	33.3	0	0.9
8	M	9	0.5	60.5	37	0.5	1.5
9	M	5	3.5	57.9	38.2	0	0.4
10	F	6	1.8	47.6	49.8	0.3	0.5
Median (min, max)			2.7 (0.5, 6.1)*	54.3 (41.6, 69.3)	41.8 (24.3, 55.2)	0.2 (0, 0.7)	0.7 (0, 3.5)
11	M	5	5.3	49.3	43.9	0	1.5
12	F	13	50	23.9	25.7	0	0.4
13	F	6	12.6	48	37.4	0	2
14	M	17	5.8	38.7	55.5	0	0
15	M	18	16.7	43.4	30.8	6.3	2.8
16	M	16	5.6	30.1	60.6	2.8	0.9
17	F	10	12.8	51.4	34.5	0.7	0.7
18	M	16	26.5	48.1	22.7	0	2.7
Median (min, max)			12.7 (5.3, 50)*	45.7 (23.9, 51.4)	36 (22.7, 60.6)	0 (0, 6.3)	1.2 (0, 2.8)

*$P \leq 0.01$ (Mann Whitney test).

eosinophils <0.5%, basophils <2%), with the exception of 2 horses with a marginal increase of neutrophils and one horse with high basophil count.

On the contrary, airway-compromised horses showed significantly higher counts for nondegenerate neutrophils. Interestingly, 2 horses also had increased eosinophils (*Horses 15* and *16*, 6.3 and 2.8%, respectively), suggesting that this is a rather heterogeneous group with varied pathophysiological conditions and/or disease states. The cytological findings of the horses included in the study are summarised in Table 2.

Tissue-specific AR expression profiles

RT-PCR analysis from one healthy horse revealed a heterogeneous expression profile for the 4 AR subtypes across the tissues explored. The RT-PCR reactions were loaded on an agarose gel (Fig 1a) and analysed quantitatively (Fig 1b). Product length, specificity and the absence of primer-dimer products were confirmed in BAL cells and cerebellum (Supplementary Fig S1).

While A_1AR could not be detected in BAL–recovered cells, this AR was expressed both in cerebellum and cortex, jejunum, caecum and eye. Exceptionally high A_1AR levels were detected in fat, in agreement with previous reports showing that this subtype is heavily expressed in this tissue, mediating most of the effects of adenosine on adipocytes (Kaartinen *et al*. 1991). A_{2A}AR could be detected in all samples, although levels were very low in cerebellum, cortex and kidney. Significant levels of this AR were identified in spleen (where it was the predominant AR type), in adrenal and in myocardium, while high levels were detected in PBMCs. Among all ARs, the A_{2B} subtype was the most commonly detected. Expression in PBMCs was very prominent, with high levels in BAL cells and cerebellum, and intermediate in cortex, aorta and adrenal gland. A_{2B}AR transcripts were less abundant in myocardium, stomach, spleen, fat and eye. In contrast, expression of the A_3AR was less common, with highest levels in PBMCs and lower ones in BAL cells, jejunum, adrenal, fat and eye. Interestingly, the expression pattern in PBMCs was similar to that of BAL-cells, but with overall expression values about 10-fold higher than BAL.

AR expression in BAL cells from airway-compromised and healthy horses

Studies examining the expression of ARs in asthmatics and COPD patients support the hypothesis of a fundamental alteration in adenosine signalling in these patients (Varani *et al*. 2006). The analysis presented here revealed A_{2B}AR as the predominant subtype in equine BAL cells, with lower levels of A_{2A}AR and marginal A_3AR expression. Therefore, we wanted to assess the expression pattern of ARs in BAL from airway-compromised horses as compared to healthy controls. Although the differences in A_{2A}AR and A_{2B}AR expression between both groups were not significant, a tendency for decreased A_{2B}AR expression in airway-compromised horses was observed (Fig 2, $P = 0.084$). This trend is in agreement with data from human with chronic airway inflammation who exhibit lower A_{2B}AR expression, suggesting a role for adenosinergic signalling in equine airway disease. Note that A_1AR was not detected in any BAL samples and that A_3AR expression levels were marginal in both groups (not shown).

Induction of IL-6 by the adenosine analogue NECA

Based on these observations, we wanted to determine whether AR activation could trigger the expression of inflammatory cytokines in BAL-cells. Because IL-6 is a major inflammatory cytokine regulated by several stimuli, we decided to investigate whether AR engagement could affect its expression in BAL cells. First, we determined IL-6 baseline expression levels in all samples relative to those of GAPDH. Figure 3a shows expression levels in nonstimulated cells from airway-compromised and from healthy controls. IL-6 mRNA abundance did not differ significantly between both groups. Furthermore, RT-PCR cycle numbers for this cytokine in both groups were close to threshold of detection (cycle 35), implying that IL-6 was minimally transcribed in resting cells from either group.

Subsequently, the cells were treated for 3 h with the nonselective AR agonist NECA and IL-6 induction reassessed (Fig 3b). While cells from healthy controls failed to upregulate this cytokine (1.54-fold, not significant), stimulation of cells from airway-compromised horses resulted in a significant increase of IL-6 transcript (2.51-fold), which was maximal at this time point. This increase in IL-6 expression in NECA-stimulated BAL cells from diseased horses was significantly greater than that of control horses ($P<0.01$). This suggests that BAL-cells from affected animals differentially react to the endogenous nucleoside adenosine.

Differential induction of IL-6 in BAL-recovered cells by AR-subtypes

The A_{2A}AR subtype has been linked with anti-inflammatory functions at several levels and it has been suggested to be a critical part in negative

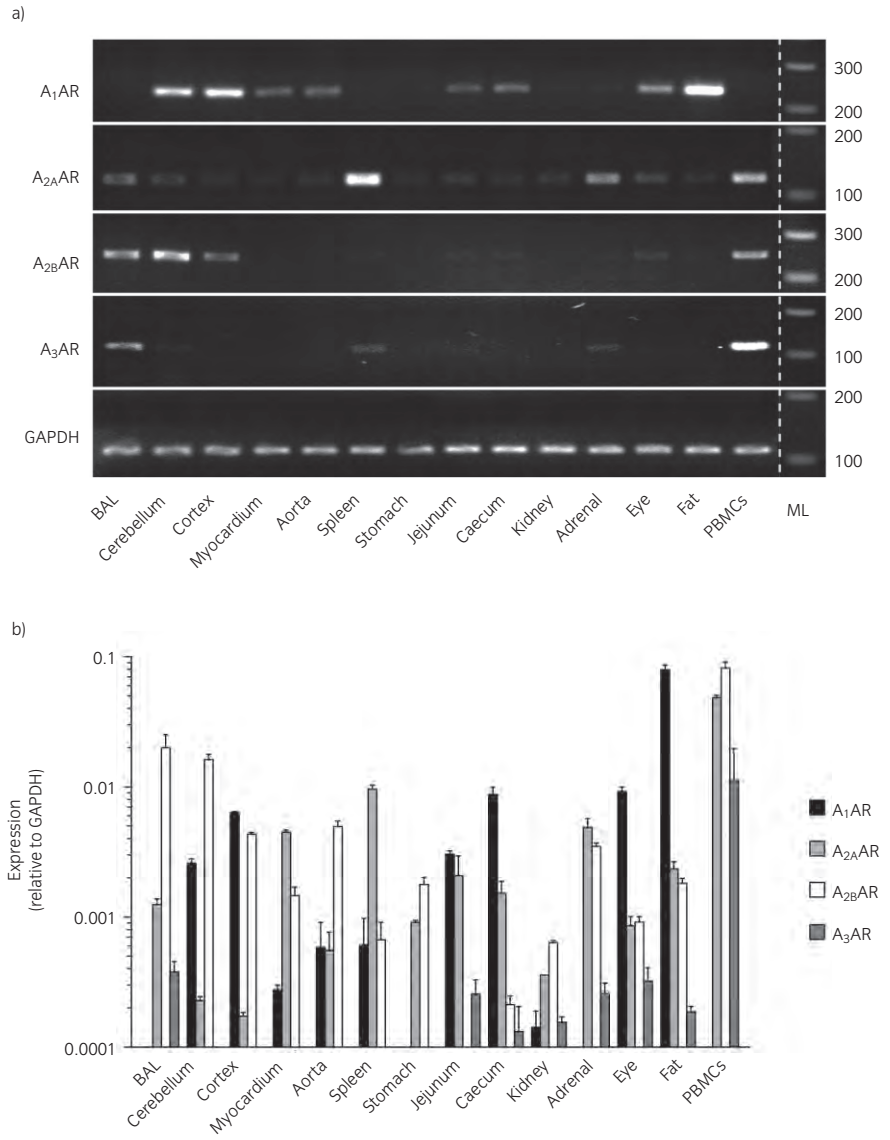

Fig 1: Expression profiles of ARs in tissue samples from a single healthy horse, assessed by RT-PCR. a) Finalised reactions (cycle 45) were loaded in an agarose gel for side by side for comparison. GAPDH reactions are included as a loading control. ML: DNA molecular ladder, sizes are given in base pairs. b) Quantitative analysis. The values are relative to GAPDH and indicate mean and standard deviation of 3 independent measurements.

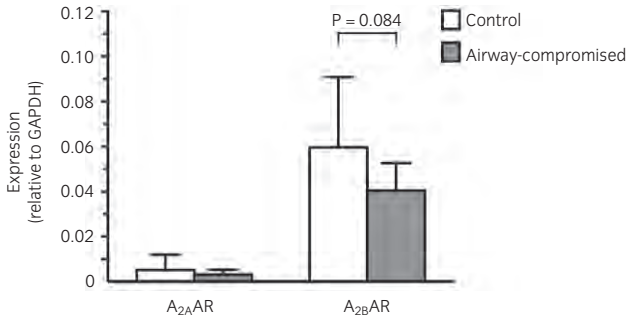

Fig 2: AR expression in BAL-samples from airway-compromised and control horses, by RT-PCR. Expression values are relative to GAPDH and indicate mean and standard deviation of 3 independent measurements (*t* test with Welch correction).

feedback mechanisms for the limitation and termination of inflammatory responses (Ohta and Sitkovsky 2001). Therefore, we wanted to assess the role of this receptor subtype in inflammatory disease in equine airways. More precisely, our aim was to determine whether activation of this AR-subtype could influence adenosinergic proinflammatory signalling in BAL-recovered cells. Therefore, we first tested whether A_{2A}AR activation could influence IL-6 expression. As expected, activation of A_{2A}AR with the selective agonist CGS-21680, at a concentration that has been shown to effectively inhibit LPS-mediated ROS production in equine neutrophils (Sun *et al*. 2007), did not induce IL-6 expression in BAL cells either from airway-compromised or healthy controls (Fig 4a). Further, we evaluated whether A_{2A}AR blockage could influence IL-6 upregulation by NECA, by treating cells with a combination of NECA plus the A_{2A}AR antagonist SCH-58261. Because this is the first study in which equine cells are exposed to SCH-58261 the concentration of 1 μmol/l was extrapolated from binding studies in rat and bovine tissues and functional assays in rabbit and porcine systems (Zocchi *et al*. 1996). While SCH-58261 did not affect IL-6 expression, the combined treatment of NECA and SCH-58261 resulted in a moderate but significant increase of IL-6 expression in healthy controls

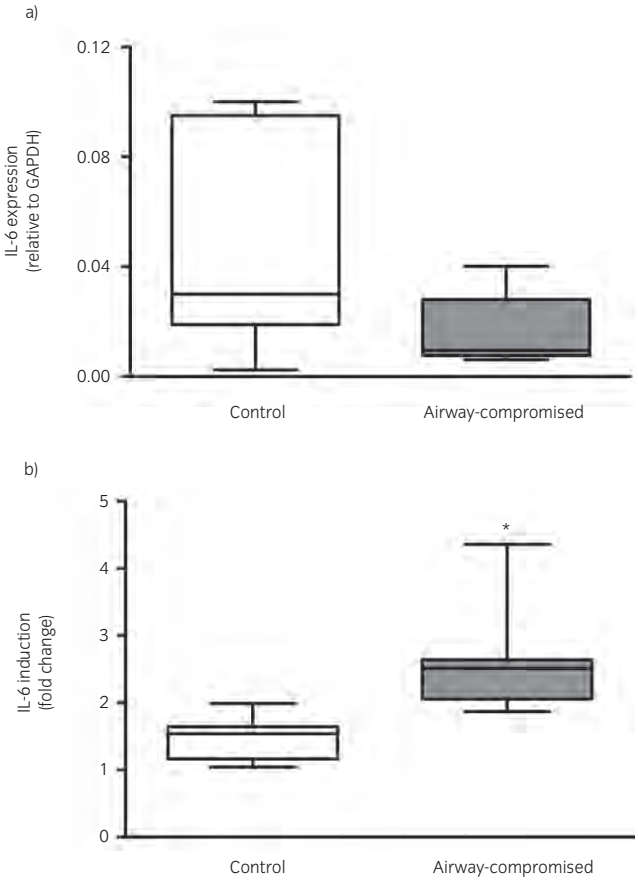

Fig 3: a) Expression of IL-6 in resting, nonstimulated BAL cells. cDNA was obtained from each horse and IL-6 abundance assessed by RT-PCR. Expression values are relative to GAPDH. b) Induction of IL-6 by NECA in BAL cells. Values for each reaction were normalised against GAPDH and fold changes calculated in relation to basal values for each horse (resting cells). Whiskers represent 10 and 90 percentiles. *: P≤0.01 vs. basal values and between groups (Mann-Whitney U test).

(median 5.28; s.d. = 1.96), when compared to NECA alone. Similarly, A_{2A}AR blockage in airway-affected horses resulted in prominent increases in IL-6 induction by NECA, reaching up to 29-fold upregulation (median = 13.04; s.d. = 7.84). In addition, IL-6 upregulation by combined NECA and SCH-58261 treatment was significantly different between both groups (Fig 4a). Altogether, this data shows that activation of A_{2A}AR in BAL-recovered cells can modulate proinflammatory signalling by other ARs, particularly in airway-affected horses.

Finally, we examined whether this differential IL-6 induction by AR activation could be associated with the changes in BAL cytology. Indeed, we detected a correlation between neutrophil count and IL-6 induction by NECA plus SCH-58261 for all 18 horses included in this study (Fig 4b), suggesting a causal relationship between the neutrophil number and AR-dependent IL-6 induction. A similar correlation was observed with IL-6 induction by NECA (not shown).

Discussion

In this study we first characterised the expression of adenosine receptors in BAL samples, as compared to other tissues. Besides the tissue-specific variations that hint towards divergent adenosinergic pathways in different organs and systems, whose interpretation exceeds the scope of this study, we observed a remarkable similarity between the AR profiles of BAL-cells and PBMCs. A_{2B}AR was predominantly expressed in both of these cell types, followed by A_{2A}AR and lower A_3AR levels. This analogy and the fact that neutrophils express primarily A_3AR (Varani *et al*. 2009) insinuate that lymphocytes and macrophages are the predominant AR-expressing cells in BAL (together they represent over 90% of BAL cells). Furthermore, these observations suggest that adenosine could play an immunomodulatory function in the airways in the horse.

In line with this hypothesis, we show enhanced reactivity of BAL-cells from airway-compromised horses upon *ex vivo* AR stimulation, thereby establishing a link between adenosine signalling and airway inflammation in this species. RT-PCR analysis revealed increased expression of IL-6 upon nonselective AR activation with the adenosine analogue NECA in cells from airway-affected horses, while this response was absent in control animals. This amplified response to adenosine could be explained by adaptive responses of airway/BAL-cells to the inflammatory milieu. Long-term *in vitro* exposure studies have indeed shown desensitisation of ARs by adenosine in target cells (Fredholm *et al*. 2001). In COPD patients, for example, elevated concentrations of adenosine and other inflammatory mediators in the peripheral lung tissue correlated with downregulation of the A_{2B}AR and with increased densities of the A_{2A} and A_3AR (Varani *et al*.

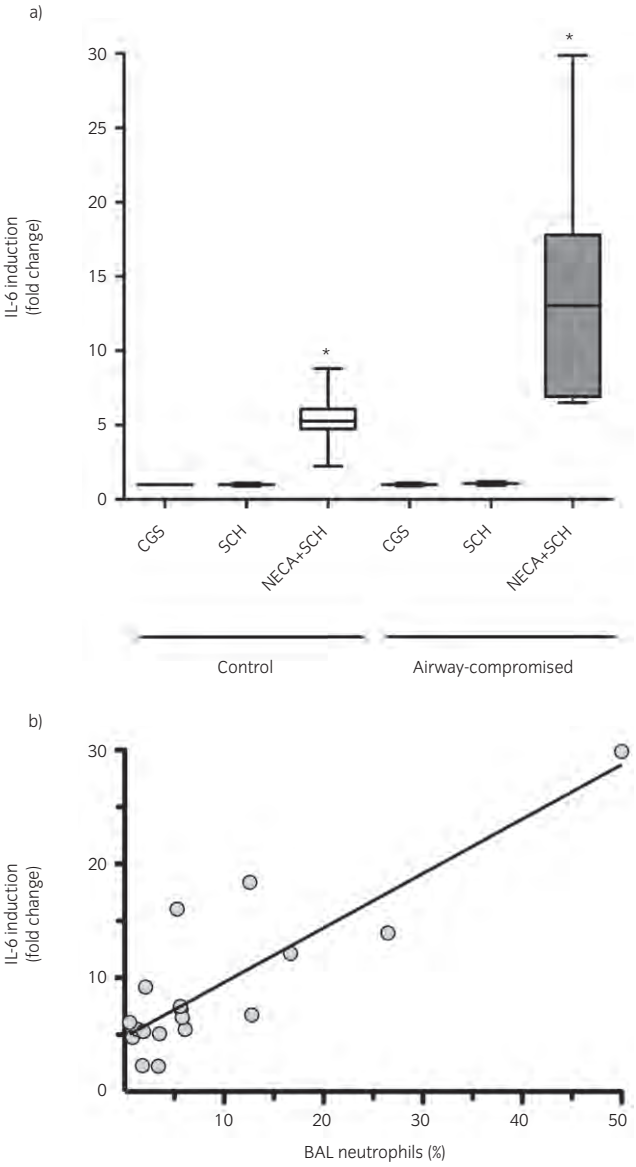

Fig 4: a) Effect of A_{2A}AR blockage on NECA-mediated IL-6 induction. Fold changes are calculated in relation to basal values for each horse (resting cells). Whiskers represent 10 and 90 percentiles. *: P≤0.01 vs. basal values and between groups (Mann-Whitney U test). b) Correlation between neutrophil percentage and IL-6 induction by NECA plus SCH-58261 for all 18 BAL samples. Spearman correlation coefficient r = 0.66, P≤0.01.

2006). In line with that report we detected a trend towards a decrease in A_{2B}AR in airway-affected horses, which was not significant (possibly because of the heterogeneity in AR expression, particularly among healthy horses). A systematic analysis of AR expression by cell type in a larger sample group would be necessary to confirm of this trend.

The exaggerated response of airway-affected horses can also be explained by the alterations in their BAL cytology. In fact, we identified a correlation between percentage of neutrophils in BAL and cell reactivity to adenosinergic proinflammatory signalling. This observation, however, does not allow to establish whether IL-6 upregulation depends on neutrophil count, or, on the contrary, whether higher neutrophils in BAL are the result of increased proinflammatory AR signalling with elevated levels of IL-6 and probably of other neutrophil chemotactic factors.

IL-6 is a multifunctional cytokine that has been associated with inflammation and the development of immune responses. Although initially considered a surrogate marker of inflammation (similar to IL-1β and TNF-α) it is now clear that IL-6 can also influence the effector functions of various $CD4^+$ T cell subsets. IL-6 inhibits Th1 differentiation and promotes Th2 differentiation during early $CD4^+$ T cell activation, and also induces the differentiation of Th17 effector cells in the presence of TGF-β (Mangan *et al*. 2006). IL-6 also inhibits regulatory T cell development, most likely by suppressing Foxp3 expression (Dominitzki *et al*. 2007). Its role in promoting Th2 and Th17 differentiation, along with inhibiting regulatory T cell activity, suggests that IL-6 might play a role in the onset and/or progression of diseases associated with these types of immune responses, including RAO. Interestingly, engagement of proinflammatory ARs has been linked to increased release of IL-6 from epithelial cells, astrocytes and fibroblasts (Sitaraman *et al*. 2001; Zhong *et al*. 2005). In addition, a recent study using Calu-3 human epithelial cells demonstrated that extracellular adenosine induces a robust release of IL-6, establishing a connection between adenosine and neutrophil infiltration in the airway lumen (Sun *et al*. 2008b).

Activation of A_{2A}AR has been associated to anti-inflammatory effects, including modulation of neutrophil and mast cell activation and degranulation, downregulation of oxidative species and adhesion molecules and inhibition of cytokine release (Lappas *et al*. 2005). In the lung, activation of the A_{2A}AR was linked to downregulation of inflammation in models of allergic asthma (Koshiba *et al*. 1999; Fozard *et al*. 2002). A_{2A}AR activation also attenuated IL-6 levels associated with inflammatory lung injury in a rat model for cardiopulmonary bypass (Mohsenin *et al*. 2007) and recent evidence indicates this AR can modulate inflammatory signals in the horse. For example, activation of the equine A_{2A}AR in a heterologous system was shown to inhibit the proinflammatory factor NF-κB (Sun *et al*. 2008a). Also, engagement of A_{2A}AR on LPS-activated equine peripheral blood monocytes led to a decrease in expression of COX-2 and TNF-α and upregulation of IL-8 and IL-10, implying a role of this receptor in the modulation of inflammatory signals (Sun *et al*. 2010). Here, we investigated for the first time the role of A_{2A}AR in the airways. Selective blockage of the A_{2A}AR in BAL-cells with SCH-58261 allowed us to distinguish between the effect of this receptor subtype and the composite effect of all ARs by NECA, thereby revealing the existence of functional A_{2A}AR in equine airways. Because activation of nonA_{2A}ARs in BAL cells with NECA plus SCH-58261 resulted in exaggerated IL-6 transcription, it can be reasoned that A_{2A}AR downregulates the induction of the proinflammatory IL-6 induction by the other ARs expressed in these cells (mainly A_{2B}AR). This effect on the expression of this proinflammatory cytokine supports the idea of selective pharmacological activation of A_{2A}AR as a therapeutic principle in airway inflammation in the horse.

In summary, this study shows that IL-6 is rapidly induced in BAL-cells of airway-compromised horses in response to adenosine exposure, probably through A_{2B}AR activation and that this effect can be modulated by A_{2A}AR. Further analysis of the effect of A_{2A}AR activation on other inflammatory stimuli will be required for a more complete assessment of the therapeutic potential of selective AR activation in the horse.

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Authors' declaration of interests

No conflicts of interest have been declared.

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Manufacturers' addresses

[1]Streuli Pharma AG, Uznach, Switzerland.
[2]Virbac AG, Glattbrugs, Switzerland.
[3]Invitrogen, Carlsbad, California, USA.
[4]Sigma-Aldrich, St. Louis, Montana, USA.
[5]Macherey-Nagel, Düren, Germany.
[6]Applied Biosystems, Carlsbad, California, USA.
[7]Ficoll-Paque PLUS, GE Healthcare Biosciences, Uppsala Sweden.
[8]Bio-Rad, Richmond, California, USA.
[9]SPSS Inc., Chicago, Illinois, USA.

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Supporting information

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Fig S1. Primer specificity. RT-PCR reactions were performed with the indicated primers and BAL or cerebellum cDNA. The reactions yielded single bands at the expected sizes and no unspecific secondary bands are observed. A reaction for GAPDH amplification without DNA is shown (-). ML: DNA molecular ladder, sizes are given in base pairs.

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8-Curriculum Vitae

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Education

- 2008-present — PhD student. Graduate School for Cellular and Biomedical Sciences, University of Bern, Switzerland
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  Martin-Luther-University Halle-Wittenberg, Germany
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Work and Research Experience

- 08/2008-07/2012 — Institute of Pharmacology and Toxicology, Vetsuisse-Faculty.
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Publications

1. **Zhang L**, Franchini M, Wehrli Eser M, Dip R. Enhanced IL-6 transcriptional response to adenosine receptor ligands in horses with lower airway inflammation. Equine Vet J. 2012 Jan;44(1):81-7.
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Declaration of Originality

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I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

Place, date Signature

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